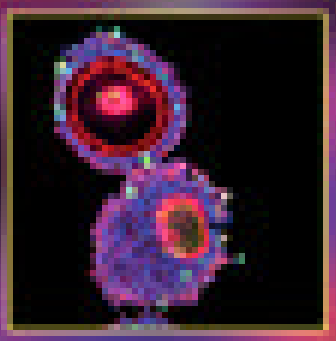
# Principles and Practice of Fertility Preservation



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# Principles and Practice of Fertility Preservation

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This book is dedicated to our courageous patients fighting cancer

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# Foreword

One of the first and most poignant cases that impressed on me the importance of fertility preservation was that of a young policewoman. She was already engaged to be married when she was diagnosed with non-Hodgkin's lymphoma. Her eagerness to preserve fertility and avoid premature menopause was so understandable, although in those days the only technology available for her was experimental. Nevertheless, she grasped the straw, saying, "It was worse to hear the treatment would sterilize me than when my doctor told me I had cancer." … She died.

The prospects for survival of young cancer patients are much better now than 20 years ago and continue to improve, but progress has often depended on more aggressive, and hence gonadotoxic, treatment regimens. Recognition of the damaging effects of chemotherapy and pelvic irradiation, and sometimes of the disease process itself, in patients of reproductive age – both women and men as well as children – is now common knowledge and has encouraged innovative technology and surgery, giving hope of preserving the potential for biological parenthood after cancer. Indeed, although the priority of medical care is to maximize the chances that patients will overcome their disease, to overlook the late effects is now regarded as neglect of a vital duty. The new developments may also help non-cancer patients, including those wishing to forestall ovarian aging, something that ought to be less controversial than often depicted in the media.

Recent growth in the field has been signaled by the launch of an international society for specialists in fertility preservation, biomedical symposia, review articles and a few books, but none as comprehensive as this volume compiled by Professors Jacques Donnez and S. Samuel Kim. The topics authored by foremost researchers and practitioners in the 43 chapters range from biology and oncology to technology and surgery, including bioethics and law. Such a large work confirms that this field has moved beyond the pioneering stage towards maturity, and it will likely be an important reference for some years to come.

*Roger G. Gosden*

# Foreword

Three decades have elapsed since the historic birth of Louise Brown in England. This momentous event, the introduction of human in vitro fertilization (IVF) as an approach to treating infertility, set the stage for hundreds of thousands of infertile couples who were ultimately to benefit from this exciting new technology. As with many medical/scientific breakthroughs, the initial process has evolved significantly from its prototype which involved a natural cycle, less than ideal monitoring and laparoscopic egg recovery. During the 30 years since IVF became a reality, new drugs and techniques have developed for follicle stimulation. Ultrasonography has come of age and is now used for follicle monitoring, egg retrieval, embryo transfer and even detection of early pregnancy. New cryopreservation techniques have enabled embryo, egg and ovarian tissue storage. Further refinements include specialized growth media for embryos, intracytoplasmic sperm injection, transdermal surgical sperm retrieval, preimplantation genetic diagnosis, egg donation and use of surrogate or gestational carriers. Couples seeking correction of infertility problems have not been the only beneficiaries of our new reproductive technologies.

The term "fertility preservation" refers to a totally new direction for these exciting technological advances. Fertility preservation applies to individuals who have deferred pregnancy for a variety of reasons, such as single women who choose to have their eggs preserved for "fertility insurance" and cancer patients about to embark on extirpative surgery, radiation or chemotherapy to cure their disease. In many instances, these women and men are neither married nor have specific plans for having offspring at the time of their impending cancer therapy. Such individuals now have a variety of options available to them which could be applied to heightening their opportunites for parenthood in the future.

Unintended consequences of our new reproductive technologies encompass profound social and ethical implications. For the cancer victim, awareness of the consequences of treatment on her future fertility must be stimulated by the oncologist during indoctrination to what may lie ahead. For young individuals, under age for providing informed consent, assent is required through guidance by both physician and parents. Requiring an invasive procedure for obtaining gametes to place in storage has its drawbacks, and the long-term storage of gametes becomes even more complex for families of individuals who succumb to their disease.

Professors Donnez and Kim's book clearly points out that the social and ethical complexities of fertility preservation for cancer patients require the collaboration of oncologists and reproductive endocrine and infertility specialists to provide the best possible information and strategic plan for each patient. The textbook unites multiple disciplines while covering basic reproductive physiology, principles of cancer therapy, age-associated issues and ethical dilemmas. It intertwines the bittersweet combination of passion for procreation and the hazards of advancing age and lethal disease. The substance and structure of this text should advance the missions of both infertility specialist and oncologist.

*Edward E. Wallach*

# Preface

Fertility preservation has become a very prominent area of interest in reproductive medicine and oncology. In the twenty-first century, fertility preservation is no longer a theoretical concept but an essential clinical discipline in medicine. Increased long-term cancer survival has intensified the need for fertility preservation strategies, as fertility is the leading quality of life issue for young cancer survivors. Although the focus of fertility preservation has mainly been limited to cancer patients in their reproductive years, its clinical relevance may well be expanded to non-cancer patients, and much broader clinical applications are expected in the future.

In the past few years, we have witnessed huge scientific and technological advances in fertility preservation methods, as well as accumulation of an enormous amount of related information and knowledge. As pioneers who have actively participated in the development of emerging technologies in fertility preservation, we felt the need to publish a comprehensive book that would reflect all aspects of this exciting new field. This book covers the full range of scientific concepts and emerging techniques, including the latest developments in oocyte cryopreservation, in vitro follicle culture and ovarian cryopreservation and transplantation.

The first section (three chapters) serves as a general introduction to the field of fertility preservation, followed by two sections (nine chapters) dedicated to cancer biology, epidemiology and treatment, as well as reproductive biology and cryobiology. In section four, fertility preservation in the male is discussed (five chapters). The following sections (19 chapters) are devoted to fertility preservation strategies in the female, divided into four categories: medical/surgical; assisted reproductive technology (ART); ovarian cryopreservation and transplantation; and in vitro follicle growth and maturation. The last two sections (seven chapters) address future technologies and ethical, legal, moral and religious issues related to fertility preservation.

We are confident that this book will provide a theoretical and practical guide for scientists, embryologists, nurses and clinicians working in reproductive medicine and oncology. In addition, it will be a valuable resource for anyone wishing to learn more about this field for patient care or research purposes.

# Acknowledgements

We, Jacques Donnez and S. Samuel Kim, would like to express our gratitude to Cambridge University Press and its editors, Nisha Doshi, Alice Nelson, Joanna Endell-Cooper and, in particular, Nick Dunton, who supported the idea of publishing this book. We greatly appreciate the time and effort of all contributing authors. Indeed, publication of this book would not have been possible without their expertise, knowledge and enthusiasm. We would also like to acknowledge the devoted members of the International Society for Fertility Preservation (ISFP), who are the future in this field. Sincere appreciation is extended to Norma Turner and Marie-Madeleine Dolmans for their dedication to the ISFP.

S. Samuel Kim is grateful to each member of the IVF team at the Center for Advanced Reproductive Medicine, the University of Kansas Medical Center, for their deep and enduring dedication, and to Carl Weiner, my department chair, for his advice and encouragement. I am indebted to Roger Gosden, my mentor and best friend, for his keen insight, relentless support and wisdom. My heartfelt thanks go to my beloved family, Kris, Jean, Melissa, Derek, Monica and my mother.

Jacques Donnez would like to thank all the members of his clinical and research teams at the Université Catholique de Louvain, who work tirelessly in pursuit of scientific advancement and clinical excellence and understand that "there is no life without pressure." I am also eternally grateful to my wife, children and grandchildren for their unwavering love and support.

Section 1 Introduction

Chapter 1

# The evolution of ART

Peter R. Brinsden

To understand science, it is necessary to know its history.
*Auguste Compte (1798–1857)*

It could be said that the first instance of assisted reproductive technology (ART) was when an eminent surgeon, John Hunter (1728–93) of London (Figure 1.1), assisted a woman in becoming pregnant by taking a semen sample produced by her husband, who had hypospadias, and inseminated her with that specimen. This was an "assisted conception," although it is not strictly within the definition of the present-day ARTs, which involve the manipulation of sperm, oocytes and embryos in vitro and include:

- in vitro fertilization (IVF)
- intracytoplasmic sperm injection (ICSI)
- gamete intrafallopian transfer (GIFT) – now rarely practiced
- zygote intrafallopian transfer (ZIFT) – now rarely practiced
- oocyte and embryo donation
- cryopreservation of sperm, oocytes and embryos
- gestational surrogacy
- in vitro maturation of oocytes
- pre-implantation genetic diagnosis.

**Figure 1.1** John Hunter (1728–93). The first reported person to successfully perform artificial insemination in a human. See plate section for color version.

However, although John Hunter's treatment of his patient was one of the first instances of outside interference with the human reproductive process, man's interest in fertility and conception in both animal species and in humans goes back thousands of years.

As early as the fifth century BC, Hippocrates (*c.* 460–370 BC), who is commonly thought of as the "father of medicine," believed that both males and females produced the "liquor" which blended within the woman's body and created babies. Some 100 years later, Aristotle (384–422 BC) proposed the theory that children are the product of "the mingling of male and female seed." This firmly opposed the then prevailing theory that children were from the male "seed" and women were merely the receptacle for the child. This latter idea prevailed until the sixteenth century, when William Harvey (1578–1657) (Figure 1.2), having studied the behavior and fertility of the King of England's herd of deer, wrote *De Generatione Animalium* in 1651, which described the egg as being

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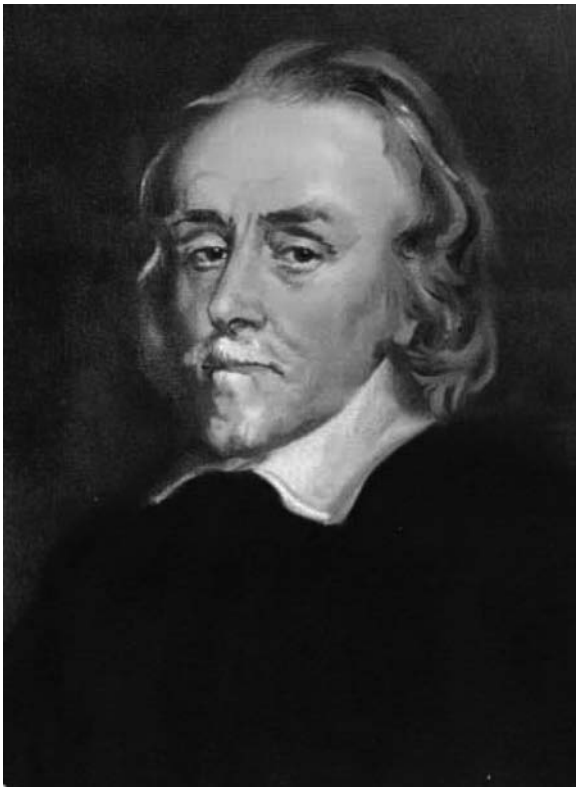

**Figure 1.2** William Harvey (1578–1657). The first person to describe the egg as responsible for the production of all creatures. See plate section for color version.

**Figure 1.3** Anton van Leeuwenhoek (1632–1723). The first person to study animal and human sperm under microscopes, which he constructed himself. See plate section for color version.

responsible for the production of all creatures. It was from this research that his famous expression "*ex ovo omnia*" [from the egg everything] arose. It was from this time onwards that the science of animal and human reproduction really began to develop. However, it was not really until the development of the optical microscope that researchers were able, for the first time, to study sperm, oocytes and, later, fertilization.

Anton van Leeuwenhoek (1632–1723), a Dutch draper and amateur scientist (Figure 1.3), was fascinated by the potential of the new science of microscopy. He built his own microscopes and, among many other specimens, he studied sperm of different animal species; in 1677 he reported his findings to the Royal Society in London. He believed that each sperm was the beginnings of an individual animal or human and, if it was "nourished" in the womb, it would produce the next generation. This went against the prevailing opinion at the time that the woman produced the seed and the male merely produced the fertilizing power to produce offspring.

Dalenpatius in 1699 stated that he could see a miniature human within a single sperm, and the idea that humans were pre-formed within a sperm prevailed for more than a century, even though this report was later found to be an hoax and Dalenpatius the fictitious name of the perpetrator of the hoax. It was Reinier de Graaf (1641–73) who first described the development of ovarian follicles – later to become known as Graafian follicles in his honor – but he never discovered oocytes within the follicles. He also supported the work of fellow Dutchman, van Leeuwenhoek and was aware of the importance of his work on microscopes. De Graaf died at the early age of 32 years.

Lazzaro Spallanzani (1729–99) (Figure 1.4), an Italian scientist, studied the behavior of semen microscopically and performed the first known attempts at insemination of a dog. He is also credited with the very earliest attempts at IVF in experiments with frogs; he is also said to have been the first to freeze and thaw sperm in 1776.

In 1826, Karl Ernst von Baer (1792–1876) (Figure 1.5) first identified oocytes in the ovaries of a bitch. He also finally established that mammals develop from oocytes and reported on organogenesis of early

**Figure 1.4** Lazzaro Spallanzani (1729–99). Performed the first known insemination of a bitch, first in vitro fertilization with frogs and was the first to successfully freeze and thaw sperm.

**Figure 1.5** Karl Ernst von Baer (1792–1876). The first person to identify oocytes in the ovaries of a bitch and to identify that mammals develop from oocytes. Credited as being "The founding father of modern embryology."

mammalian embryos. Von Baer is credited with being the "founding father of modern embryology."

In the mid nineteenth century, extensive research was carried out on reproduction by a number of researchers who reported their observations on the process of fertilization in primitive organisms; in particular, Henry Nelson (1852), Newport (1853), van Beneden (1854) and Hertwig (1876). Nelson observed the penetration of ascaris oocytes by spermatozoa; Newport made similar observations in amphibians, while both van Beneden and Hertwig are credited with the first observations on fertilization in mammals.

It is probable that Walter Heape (1855–1929), a physician and professor at the University of Cambridge, England, was the first scientist to successfully transfer embryos into rabbits in the early 1890s. Only one pregnancy and delivery was reported, but this experiment showed for the first time that it was possible to remove embryos from one animal and transfer them to another, without interfering with their development.

In their reviews on the early history of IVF, both Bavister [1] and Clark [2] give the opinion that 1951 was probably the "critical boundary" defining the beginning of the modern era of IVF. Both Colin "Bunny" Austin (1914–2004) and M. C. Chang (1908–91) discovered the need for spermatozoa to undergo capacitation and the acrosome reaction before they are able to penetrate the zona pellucida of the oocyte. Later, in 1963, Yanagimachi and Chang, were able to achieve the first live births after transfer of hamster oocytes fertilized in vitro using spermatozoa capacitated in vitro [3]. Work continued, more or less successfully, over the next decade attempting to achieve fertilization in vitro and live births of various other mammalian species.

Some of the earliest observations on fertilization of human oocytes were made by Robert Edwards (1925–) and published in 1965 in a landmark paper: "Maturation in vitro of human ovarian oocytes" [4]. It was not possible for Edwards to progress further with efforts to achieve IVF of human oocytes for clinical use without close collaboration with clinical colleagues, who were able to provide a supply of human oocytes – usually from patients having ovarian wedge resections for polycystic ovary disease. It was the need for Edwards to be able to obtain these supplies of pre-ovulatory human oocytes that brought him and gynecologist Patrick Steptoe (1913–88) together in 1968.

Patrick Steptoe became known to Robert Edwards because he had brought laparoscopy, whereby the female pelvic organs could be visualized by a relatively minor operation, to England from Europe where Steptoe had studied it under both Raoul Palmer (1940–95) and Hans Frangenheim (1920–2001). On his return to England, he further developed the technique of laparoscopy, and shortly afterwards he wrote his famous short textbook *Laparoscopy in Gynaecology* in 1967 [5]. His first major paper: "Laparoscopy and ovulation" followed in 1968 [6]. Shortly afterwards, he further developed the laparoscopic technique to enable aspiration of oocytes from follicles under direct vision.

Robert Edwards started his career in reproductive biology at the Institute of Animal Genetics and Embryology, Edinburgh, in 1951, having just been demobbed

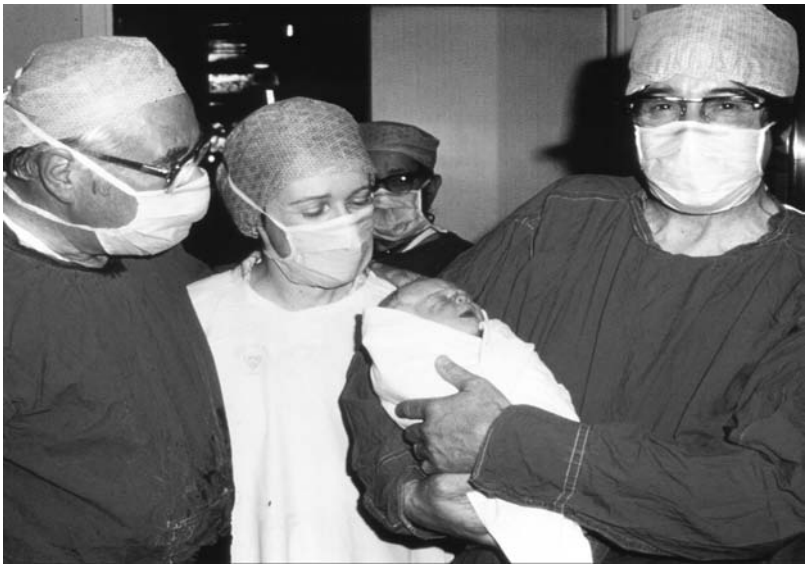

**Figure 1.6** Patrick Steptoe and Robert Edwards at the birth of the world's first in vitro fertilization conceived baby – Louise Brown – on July 25, 1978. Courtesy of Bourn Hall Clinic. See plate section for color version.

from the British Army. There, under the supervision of Professor Alan Parkes, he did his PhD on reproductive genetics. He moved on to the Institute of Medical Research, London, and then to the University of Cambridge in 1963 to join two well-known researchers in reproductive physiology: Professors Alan Parks and "Bunny" Austin. There Edwards continued his work on immunology and oocyte maturation. He then spent a short time in the USA at Johns Hopkins University, where he collaborated with two other "greats" in the field of human ART, Drs. Howard and Georgeanna Jones. On his return to Cambridge, he continued his work on human oocytes. In 1968, Edwards, who had heard about Steptoe's work in retrieving pre-ovulatory human oocytes laparoscopically, contacted him and met with him at a Royal Society of Medicine meeting in London. They quickly struck up a working relationship and friendship which, between 1968 and 1978, led them to further develop the techniques of human IVF and applied it to the clinical treatment of intractably infertile women.

Steptoe and Edwards soon started to produce landmark papers together – in 1969: "Early stages of fertilisation in vitro of human oocytes and matured in vitro" [7]; and also in 1969: "Identification of the midpiece and tail of the spermatozoon during fertilisation of human eggs in vitro" [8]; and in 1970: "Laparoscopic recovery of preovulatory human oocytes after priming of ovaries with gonadotrophins" [9]. They also carried out the first treatment cycles of oocyte recovery with tubal insemination (ORTI), as they called the procedure, which was later to become known as gamete intrafallopian transfer (GIFT).

Between the years 1968 and 1978, while they were working closely together, Steptoe was in Oldham, Lancashire, England, working in a National Health Service hospital, and Edwards was at the University of Cambridge. When the clinical treatment of infertile women started, there followed a number of very difficult years in which none of the first 40 patients they treated by IVF and embryo transfer (ET) became pregnant. In 1976 they did achieve their first pregnancy following transfer of a single blastocyst, but this subsequently turned out to be an ectopic pregnancy [10]. After 102 failed embryo transfers, including the one ectopic pregnancy, Leslie Brown was treated and subsequently became pregnant following her first embryo transfer. This was achieved in a "natural" IVF cycle, with no stimulation; one oocyte was collected and a single eight-cell embryo was transferred. There followed a difficult pregnancy for Mrs Brown, but her baby, Louise Brown, was delivered by cesarian section on July 25, 1978 (Figure 1.6). Much to everyone's relief, baby Louise was found to be a perfectly normal, fit and healthy infant. This momentous achievement was announced with a simple publication as a letter in the *Lancet*: "Birth after reimplantation of a human embryo" [11]. The arrival of Louise Brown was heralded as "The baby of the century." This was in spite of considerable criticism and opposition by clinical and

**Figure 1.7** World's first in vitro fertilization conference, held at Bourn Hall in 1981. Courtesy of Bourn Hall Clinic.

scientific colleagues, the lay press and by many representatives of different religious faiths.

Work on human IVF was also being conducted elsewhere in the world, particularly in Melbourne, Australia, where the team of Professor Carl Wood and Dr. Alan Trounson achieved the birth of the world's fourth IVF baby, Candice Reed, in June 1980 [12]. At the same time, Drs. Howard and Georgeanna Jones had been working in Norfolk, Virginia, USA, and Elizabeth Carr, the first US in-vitro conceived baby, was born on December 28, 1981 [13].

In England, meanwhile, Patrick Steptoe and Robert Edwards were unable to continue their work on human IVF, since neither the UK's National Health Service nor any of the Universities or the Medical Research Council were willing to provide funding to help them to continue their work. They eventually found Bourn Hall, an old Jacobean manor house in the Cambridgeshire countryside, where they founded the World's first IVF treatment and research center – Bourn Hall Clinic – which opened in September 1980 (Figure 1.7). There, Steptoe and Edwards continued their research and, by 1986, they had achieved 500 live births [14]. Steptoe and Edwards achieved a number of distinguished national and international awards over the next 3 years, but, in 1988, Patrick Steptoe fell seriously ill with prostate cancer and died on March 21, 1988. Robert Edwards continued to work as Scientific Director of Bourn Hall and as Editor of the newly formed journal *Human Reproduction*, which he cofounded. In 1994, he retired from working at Bourn Hall.

In the early years following the first IVF births in England, Australia and the USA, other teams were successful in achieving births: in 1982 in France from the group of Professors Frydman and Testart; and in Sweden from the group of Professor Lars Hamberger. In 1982, in England, both Dr. Brian Lieberman's group and Professor Ian Craft's group also achieved live births, followed by, in Austria, Professors Feichtinger and Kemeter, and later that year births also occurred in Finland, Germany and the Netherlands.

An interesting historical point is the suggestion that the world's second IVF baby was achieved in India, following work by Dr. Subhash Mukhopadhyay. A baby was born on October 3, 1978 following IVF and ET, but his achievement was never officially recognized; indeed, he was derided by his colleagues and officials in India at the time. He eventually committed suicide in 1981. However, following a close investigation of his claim some 27 years later, he was officially accepted as being the first Indian and second in the world to achieve a live birth following IVF.

In 1983, the first IVF baby was born following the transfer of frozen–thawed embryos in Australia [15]. Oocyte donation, as a treatment option in IVF programs, also developed from about the mid 1980s, with the first successful live birth reported from Australia [16]. Originally developed to treat women with premature menopause, this was extended to treat women with inherited diseases and, increasingly now, is being used in the treatment of women in their mid to late 40s, or even older, to help them to have children

late in their lives. In a few countries, treatment using donated embryos has been permitted and, in some countries, treatment using gestational surrogates has become available to treat women without a uterus or with other reasons meaning that they are unable to carry a child. Utian *et al*. published the first report of an IVF birth in the USA through gestational surrogacy in 1985 [17].

The need to develop more "user friendly" techniques to obtain oocytes, other than by the relatively invasive technique of laparoscopy, was developed by Lenz and Lauritsen in 1982, who described the technique of abdominal ultrasound-guided needle oocyte recovery [18]. Gleicher *et al*. further developed this technique in 1983, approaching the ovaries transvaginally but using an abdominal probe [19]. Later, in 1985, Mats Wikland in Sweden developed the now almost universally used transvaginal ultrasound probe-guided needle aspiration of pre-ovulatory follicles [20]. The techniques of intrauterine insemination (IUI) and GIFT also developed over the following years as a more simplified variant of standard IVF.

Over the years since the beginning of human IVF, many changes have occurred in ovarian stimulation protocols for IVF. The major developments have been:

- 1970s
  - natural cycle IVF
  - clomiphene alone
- 1980s
  - clomiphene + urinary human menopausal gonadotropin (HMG)
  - gonadotropin-releasing hormone (GnRH) agonists + urinary HMG
  - "flare" protocol
  - ultra-short and short protocols
  - long luteal phase or follicular-phase start long protocols
- 1990s
  - GnRH agonist + urinary-follicle stimulating hormone (FSH) intramuscularly (im)
  - GnRH agonist + high purity FSH subcutaneously (sc)
  - GnRH agonist + high purity HMG (sc)
  - GnRH agonist + recombinant human follicle stimulating hormone (rhFSH) (sc)
- Late 1990s–2000+
  - GnRH antagonists + rhFSH ± recombinant human luteinizing hormone (rhLH)
  - recombinant LH
  - recombinant human chorionic gonadotropin (hCG)
  - "fill by mass" versus IU recombinant FSH
  - "patient friendly" sc injections and use of "pens" for injection

As can be seen above, stimulation protocols have undergone many changes. In the early days, IVF was conducted in natural cycles or with clomiphene-only stimulation. It was in 1984 that Porter *et al*. in London first developed the use of GnRH agonists in IVF stimulation protocols to prevent premature LH surges [21], which, over the following years, became the "gold standard" for use in IVF stimulation protocols; indeed, it remains so for many practitioners. Introduction of the GnRH antagonists in stimulation protocols, first reported by Frydman *et al*. in 1991 [22], increasingly has become used, allowing a more "natural" cycle and being more "patient friendly," since treatment does not last as long as do GnRH-agonist protocols. Also from the mid 1990s, there were major developments in the production and use of gonadotrophins. These were produced originally from human menopausal urine and injected intramuscularly. High purity FSH and HMG were a great improvement and could be injected subcutaneously by patients themselves after training. Also from about the mid 1990s, recombinant gonadotrophins were developed, producing the purest FSH, LH and hCG. It was in 1992 that Germond *et al*. [23] and Devroey *et al*. [24] reported the first pregnancies using the new recombinant FSH (rFSH).

More recently still, there has been an enthusiasm for the concept of "IVF lite." This has introduced the concept of a milder stimulation strategy for IVF in order to reduce the risk of complications of stimulation, particularly of ovarian hyperstimulation syndrome. It is also thought to improve the chance of implantation by reducing interference of the development of the endometrium that may occur in some high dose gonadotrophin stimulation protocols.

In vitro fertilization was never really successful in the treatment of severe male factor infertility, and techniques were developed to try to improve the outcome for men with this diagnosis, for whom the

only real option then was the use of donor sperm. In 1987, Laws-King *et al*., in Australia, first reported the microinjection of spermatozoa under the zona pellucida of oocytes [25]. However, it was not until 1992 that the first pregnancy after ICSI of oocytes with single spermatozoa was reported by Palermo *et al*. of the Free University of Brussels [26]. For the first time, this technique allowed men with the most intractable infertility problems to achieve pregnancies with their partners. This treatment option has been the most important development in ART since human IVF first started. Most ART units worldwide now treat 40–50% or more of their ART cycles by ICSI, and some even advocate using it for all ART cycles.

Other firsts include, in 1989, Handyside and colleagues from London who first showed it is possible to take a single blastomere from an embryo, perform pre-implantation genetic diagnosis (PGD) and to sex the embryo by DNA amplification [27]. This technology has led to the development of a whole new subspecialty of techniques used to diagnose not only the sex of an embryo but to detect a multitude of genetic abnormalities, including single gene defects, and also to perform screening for aneuploidy. In 1990, Verlinsky *et al*. reported the first polar body biopsy, with a subsequent embryo transfer and pregnancy [28]. This has proved to be most useful in countries which do not allow embryos to be manipulated or biopsied, such as Germany and Switzerland.

In 1991, Cha *et al*. developed the technique of in vitro maturation (IVM) of oocytes and reported their first pregnancy using this technology [29].

Silber *et al*. in 1994 reported the first cases of testicular sperm extraction (TESE) combined with intracytoplasmic injection [30] for men with obstructive and non-obstructive azoospermia. Other landmark developments were made in the diagnosis of male factor infertility, particularly in 1996 when Reijo *et al*. showed that some men with severe oligoasthenozoospermia had deletions on the Y chromosome [31]. The whole understanding of male factor infertility has developed dramatically in the last 12–15 years.

The first autologous transplantation of frozen–thawed ovarian tissue was conducted by Oktay *et al*. in 2001 [32] and, in 2004, Donnez *et al*. reported the first live birth after orthotopic transfer of frozen–thawed ovarian tissue [33]. These advances will do much in the future to improve the reproductive outcomes for young women who require chemotherapy or radiotherapy for malignancies.

One of the major hurdles still to be overcome by clinicians and scientists practicing the ARTs is to reduce the number of multiple pregnancies created. It is considered now to be unacceptable that some 40–50% of children born as a result of IVF and related procedures are from multiple births, with the consequent major increase in complications, both for the babies and for the mothers. There is a very positive move now towards making the majority of ETs in an IVF program single embryo transfers – be it at day 2, 3 or blastocyst stages. This change in practice is largely being led by the Northern European countries, where multiple rates have been reduced to $<$10% and even, in some practices, to $<$5% [34]. However, in certain countries, transfer of four or more embryos is occurring in some 25–35% of cycles, producing twins in 25–35% of cycles and triplets in anywhere between 2.7 and 5.7% of deliveries. This is in spite of large numbers of fetal reductions being performed. However, these alarming figures are slowly reducing, year on year.

One of the most important developments in ART worldwide during the last 32 years has been the evolution of guidelines or regulatory systems to govern the practice of the ARTs. The state of Victoria, in Australia, was the first state to pass legislation on IVF in 1984; this became known as the "Infertility (Medical Procedures) Act 1984." The UK was the first country to develop a full regulatory process and regulatory body. This started with an initial review by a Government appointed body, which produced a report in 1984 known as the "Warnock Report." This proposed a UK regulatory system which would cover clinical and scientific practices of:

- all treatment involving the creation of human embryos outside the body
- all treatment involving donated gametes
- all storage of human gametes and embryos
- all research on human embryos

The report also recommended that all clinics providing ART services should be licensed by a regulatory authority. Following publication of this report in 1984, voluntary and then interim licensing authorities were set up to monitor ART practice in the UK. The Human Fertilisation and Embryology Act finally passed through Parliament in 1990, which led to the establishment of the Human

Fertilisation and Embryology Authority (HFEA) in 1991. This body is responsible for the licensing, regulation and monitoring all units practicing ART in the UK. The rules and regulations are set out in a "Code of Practice," which is reviewed regularly, and in 2009 the eighth edition of the Code of Practice was produced, following passage of an updated Human Fertilisation and Embryology Act in 2008 [35].

Most countries in 2010 have some form of regulation, more or less strict, while other countries have guidelines, but there are still countries that have no regulation or guidelines at all. The state of regulation and practice worldwide is summarized in a 3-yearly publication produced by the International Federation of Fertility Societies (IFFS) and published by the American Society for Reproductive Medicine (ASRM); the last edition was published in 2007 [36].

The study of fertility, both animal and human, has fascinated clinicians and scientists for more than two millennia. Research into fertility and infertility led us, via many important milestones, to being able to treat women and men with hitherto untreatable infertility by IVF and related techniques. These treatments – the ARTs – are now very well established as "mainstream" treatments, almost universally accepted and practiced. Worldwide, there are now an estimated 4–5 million babies who have been born since human IVF was first successful in1978. Although the early pioneering days of IVF are over, there is still a limitless amount of research to be done in the field of ART, particularly in genetics and stem cell research. It is also to be hoped that IVF and related ARTs will become still more simple and "patient friendly" and, in particular that they may become much cheaper, so that ART can be provided in the less developed countries, where presently infertile couples are unable to obtain treatment because of cost. It has been impossible to cover the whole story of the development of ART over so many years in one short chapter, but the present status of ART worldwide is built upon the fundamental achievements of the early scientific and clinical pioneers of our specialty. Their story bears more in-depth study and understanding, to better appreciate what we all struggle to achieve for our patients – families.

A thorough comprehension of the history of IVF would improve the depth of appreciation of challenges we are facing today, hopefully resulting in improved outcomes of future treatments [37].

Just as this book was going to press, the Nobel Prize Committee made the following announcement on the 4th of October 2010:

"Robert G. Edwards, the 2010 Nobel Laureate in Physiology or Medicine, battled societal and establishment resistance to his development of the in vitro fertilization procedure, which has so far led to the birth of around 4 million people."

The many friends and colleagues of Robert "Bob" Edwards are delighted at this very happy and hugely well-deserved accolade and send him our most sincere congratulations.

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Chapter 2

# The effect of chemotherapy and radiotherapy on the human reproductive system

W. H. B. Wallace, R. A. Anderson and D. Meirow

Cancer in childhood is rare, with approximately 1400 new cases per year in the UK, and a cumulative risk of around 1 in 500 by the age of 15 in resource-rich countries. With long-term survival rates approaching 73%, it has been estimated that by the year 2010 about 1 in 715 of the adult population will be a long-term survivor of childhood cancer [1]. Cancer is more common after puberty during the reproductive life span of men and women [2, 3], and many of these patients will be cured by combination treatment with surgery, chemotherapy and radiotherapy. Long-term survivors are nevertheless at risk of developing a number of late sequelae [4], including impaired fertility, adverse pregnancy outcomes and health problems in offspring [5–7]. Loss of fertility is one of the most devastating consequences of radio- or cytotoxic therapy for these young patients who, having overcome their disease, have expectations of a normal reproductive life.

## Normal ovarian development and follicular depletion

Current understanding of human ovarian reserve presumes that the ovary establishes several million non-growing follicles (NGFs) during the second half of intrauterine life, which is followed by a decline to the menopause when approximately 1000 remain at an average age of 50–51. With approximately 450 ovulatory monthly cycles in the normal human reproductive life span, this progressive decline in NGF numbers is attributed to follicle death by apoptosis. In a recent study, the first model of human ovarian reserve from conception to menopause that best fits the combined histological evidence has been described [8]. This model allows us to estimate the number of NGFs present in the ovary at any given age, and it suggests that 81% of the variance in NGF populations is due to age alone (Figure 2.1). Further analysis demonstrated that 95% of the NGF population variation is due to age alone for ages up to 25. The remaining 5% is due to factors other than age, e.g. smoking, body mass index (BMI), parity and stress. We can speculate that as chronological age increases, factors other than age become more important in determining the rate at which NGFs are lost through apoptosis.

There is speculation that this widely held tenet of mammalian ovarian function may require revision. A report in 2004 suggested the presence of germ stem cells in the adult mouse ovary [9], and two subsequent reports by the same group suggested the ability of bone marrow-derived cells to give rise to new immature oocytes [10, 11]. Bone marrow transplant was shown to partially restore the fertility of busulfan-treated mice [11] even though all offspring derived from the host germline. More recently, another group has identified the presence of proliferative and culturable female germline stem cells in newborn and adult mouse ovaries [12]. Strikingly, when these cells (transgenically labeled) were injected into a chemotherapy-treated ovary, they became enclosed within follicles and offspring bearing the transgene were produced. These data provide a basis for re-evaluating the regenerative capacity of the mammalian ovary and new approaches for overcoming fertility loss. While the emerging evidence thus appears to provide evidence in support of the existence of germ stem cells within the adult mouse ovary, the Wallace–Kelsey model of

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
Published by Cambridge University Press. 

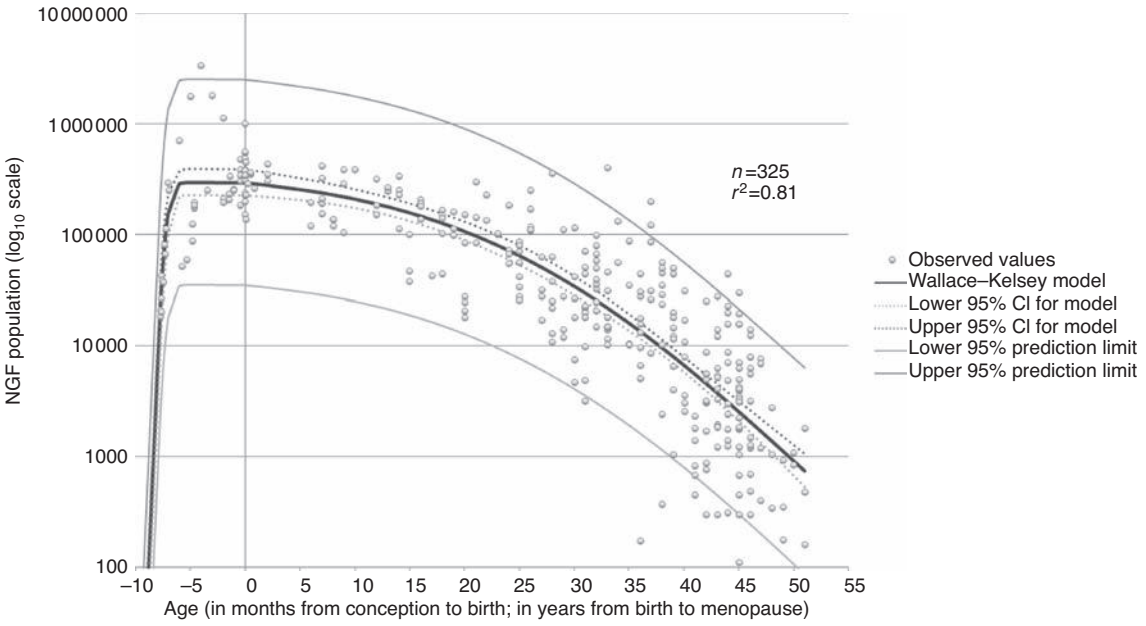


**Figure 2.1** The best model for the establishment of the non-growing follicle (NGF) population after conception and the subsequent decline until age at menopause is described by an ADC model. The model has a correlation coefficient $r^2 = 0.81$, fit standard error = 0.46 and F-value = 364. The figure shows the dataset ($n = 325$), the model, the 95% prediction limits of the model and the 95% confidence interval (CI) for the model. The horizontal axis denotes age in months up to birth at age 0 and age in years from birth to 51 years. Reproduced with permission from Wallace and Kelsey [8]. See plate section for color version.

ovarian follicle decline provides no supporting evidence of neo-oogenesis in normal human physiological aging.

## Clinical detection of chemotherapy-induced ovarian damage

Many studies use amenorrhea as a surrogate for ovarian failure, with biochemical confirmation (i.e. elevated follicle stimulating hormone [FSH] concentration) in some. While amenorrhea may of course have other causes than ovarian failure, it would seem a reasonable surrogate for population-based studies. However, an important drawback of the use of amenorrhea or elevated FSH measurements is that these only detect the endpoint of the decline of ovarian function. It would be of considerable utility to have a biochemical or biophysical marker of the number of follicles in the ovary, i.e. the ovarian reserve (Table 2.1 [13]) [14] to allow detection of lesser degrees of damage and earlier changes during the progress to ovarian failure. This would allow improved analysis of the effects of chemotherapeutic agents on the ovary and clinically allow individualized advice based on pre and postchemotherapy analysis of the ovarian reserve. Follicle stimulating hormone remains the most widely used marker of incipient ovarian failure, but it shows low sensitivity and considerable inter-cycle variability. Inhibin B is a product of the granulosa cells of growing follicles and shows a fair prediction of oocyte recovery following superovulation (the standard in vitro fertilization [IVF] -based outcome measure of the ovarian reserve). However, it is produced by the granulosa cells of large as well as small antral follicles, and thus while its physiological importance in limiting the inter-cyclic rise in FSH is undoubted, its concentration

**Table 2.1** Qualitative assessment of ovarian reserve

- Early follicle phase, follicle-stimulating hormone (FSH)
- Early follicular phase, inhibin B
- Serum anti-Müllerian hormone (AMH)
- Sonography: total antral follicle count (AFC)
- Sonography: ovarian volume
- Ovarian biopsy
- Response to ovarian stimulation

Adapted from van Rooij *et al.* [13].

declines only late in reproductive life and thus its value as a marker of loss of the ovarian reserve is limited.

Anti-Müllerian hormone (AMH) is best characterized as a product of the fetal Sertoli cells, causing regression of the Müllerian structures in the male. It was subsequently recognized that AMH is also an important product of the adult ovary, produced by the granulosa cells of smaller growing follicles [15]. Crucially, AMH secretion is only initiated at the start of follicle development and declines abruptly in the early antral stages. It is thus not a product of the dominant follicle, and only to a limited extent of FSH-recruited follicles. It would therefore be expected to decline with age and show much less variation across and between menstrual cycles than FSH or inhibin B. These characteristics have been confirmed adding to the convenience of its measurement [16]. Thus, AMH is the best currently available marker of the number of small growing follicles in the ovary [13]. It is not a direct marker of the true ovarian reserve, i.e. the number of primordial follicles, but primordial follicle number is directly related to the number of early growing follicles (although with quite wide variability).

Data are increasingly available on AMH's utility to detect chemotherapy-induced loss of ovarian reserve in survivors of childhood [17–19] and adult [20–22] cancer, and limited data from prospective studies illustrate its ability to reflect acute gonadotoxicity [20, 23]. Anti-Müllerian hormone's utility to predict long-term ovarian function and, more importantly, fertility when measured before or after chemotherapy remains to be determined.

Ultrasound can also be used to assess the ovarian reserve. As with AMH, most data derive from the context of IVF/superovulation, with the number of oocytes recovered being the primary outcome rather than short or long-term fertility. Nevertheless, both antral follicle count (AFC; the number of follicles of 2–10 mm diameter) and, to a lesser extent, ovarian volume have been explored as markers of ovarian damage during chemotherapy [20, 21]. In a prospective study of women undergoing chemotherapy for breast cancer, both AFC and ovarian volume decreased during treatment [20]. However inter-investigator variability is more important than with biochemical variables and, as these tests are also less convenient, it is likely that AMH and future biochemical developments will take precedence.

## Chemotherapy and the ovary

The ovary is susceptible to chemotherapy-induced damage, particularly following treatment with alkylating agents such as cyclophosphamide [17, 24]. Ovarian damage is drug and dose-dependent and is related to age at the time of treatment, with progressively smaller doses required to produce ovarian failure with increasing age [25, 26]. Alkylating agent dose is related to subsequent fertility in childhood cancer survivors [27].

The stockpile of primordial follicles found in the cortex of the ovaries represents the ovarian reserve. Histological studies of human ovaries have shown chemotherapy to cause ovarian atrophy and global loss of primordial follicles [28, 29]. However, these studies of human ovarian biopsies do not provide any information on the mechanism of injury. The effect of chemotherapy on the ovary is not an "all or nothing" phenomenon, and the number of surviving primordial follicles following exposure to chemotherapy correlates inversely with the dose of chemotherapy and the nature of the agent [30].

The mechanism involved in the loss of primordial follicles in response to anti-cancer therapy is not well understood. A few human and animal studies have demonstrated that chemotherapy induces damage to ovarian pre-granulosa cells [31] and that apoptosis occurs during oocyte and follicle loss [32]. In addition, injury to blood vessels and focal fibrosis of the ovarian cortex are further patterns of ovarian damage caused by chemotherapy, evidenced in ovaries of patients previously exposed to non-sterilizing chemotherapy [33]. Fibrosis and vascular changes have also been reported by others [31, 34], who examined ovarian tissue from girls treated for acute lymphoblastic leukemia (ALL). As dividing/proliferating cells are, in general, the major targets of chemotherapeutic agents, it would seem likely that the granulosa cells of growing follicles would be the most chemotherapy-sensitive cell type in the ovary. This may be the reason for the abrupt decline in serum AMH during chemotherapy [20, 23] and the cessation of menstrual bleeding for a few months after chemotherapy, due to loss of growing larger follicles. Loss of the inhibitory influence of small growing follicles on initiation of primordial follicle growth (as in some animal models [35]) will result in increased activation of the resting pool of primordial follicles and thus premature ovarian failure: it is possible that a similar mechanism contributes at least in part to the effect of chemotherapy in the human.

A reduced follicular reserve may result in premature ovarian failure (POF) and menopause many years post-treatment, even in patients undergoing chemotherapy at a very young age [24]. Significant depletion of the primordial follicle stockpile post-chemotherapy in a normally ovulating female has been demonstrated in an animal model [30].

The risk of ovarian failure in several commonly encountered malignancies and other disorders requiring chemotherapy and/or radiotherapy is presented in Table 2.2. Cyclophosphamide is widely used in combination chemotherapy regimens, and high dose cyclophosphamide (200 mg/kg) is frequently utilized as conditioning therapy before bone marrow transplantation (BMT), either alone, where recovery of ovarian function is more likely, or in combination with other chemotherapeutic agents or total body irradiation (TBI) [36].

Treatment of Hodgkin's lymphoma with MOPP (mechlorethamine, vincristine, procarbazine and prednisolone) or ChlVPP (chlorambucil, vinblastine, procarbazine and prednisolone) is associated with ovarian dysfunction in 19–63% of cases [37]. Amenorrhea is more commonly observed in older women, but long-term follow-up is necessary, as a number of young women also develop premature menopause. The BEACOPP regimen (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone) results in amenorrhea in approximately 20% of women overall, but this rises to 67% in women treated with 8 cycles of dose-escalated BEACOPP [38] with, as discussed above, age being an important factor. Amenorrhea was reported by 95% of women aged over 30 years compared to 51% in younger women. Treatment with an ABVD regimen (adriamycin, bleomycin, vinblastine and dacarbazine), which contains no alkylating agents or procarbazine, results in significantly less gonadotoxicity, especially in patients under 25 years [39]. In a recent cohort study [40] of 518 female 5-year survivors of Hodgkin's lymphoma aged 14–40 (median age: 25 years) at treatment, the Amsterdam group explored the incidence of POF before age 40. Alkylating agents, especially procarbazine (hazard ratio [HR]: 8.1) and cyclophosphamide (HR: 3.5), showed the strongest associations. Ten years after treatment, the actuarial risk of premature menopause was 64% after high cumulative doses ($>$8.4 g/m$^2$), and 15% after low doses ($\leq$4.2g/m$^2$), of procarbazine [29]. A small study demonstrated normal fecundity in women treated with ABVD [41]. The risk of POF in Hodgkin's lymphoma and breast cancer is summarized in Tables 2.2 and 2.3[42].

In cases of germ cell tumors, fertility-sparing surgery is possible in a large proportion of patients. For patients with advanced stage disease, maximum cytoreductive surgery appears to be beneficial. For patients who require postoperative chemotherapy, standard therapy involves a combination of bleomycin, etoposide and cisplatin. Although POF may occur in a small proportion of patients, 80–99% of those who undergo fertility-sparing surgery and chemotherapy can expect to maintain reproductive function [43]. In a group of young women (median age: 25.5 years) who were treated with the VAC protocol (vincristine, actinomycin, cyclophosphamide) for germ cell tumors, 13% were found to have irregular menses, 15% oligomenorrhea or amenorrhea and 8% persistent amenorrhea, although 11 of 16 who had attempted to become pregnant had been successful [44].

Chemotherapy is also used in patients with non-malignant diseases such as systemic lupus erythematosus (SLE). Pulse cyclophosphamide therapy is frequently used for active lupus nephritis or neuropsychiatric lupus. The major determinants for the development of ovarian failure in patients with SLE are age at the start of therapy and the cumulative cyclophosphamide dose (number of cycles and doses) (Table 2.2) [45]. Women with SLE and related diseases provide additional challenges for fertility preservation because of the uncertain course of their disease, and thus the poor predictability of the total dose of cyclophosphamide that will be required.

## Radiation and the hypothalamic–pituitary–ovarian axis

The ovaries may be damaged following total body, abdominal or pelvic irradiation, and the extent of the damage is related to the radiation dose, fractionation schedule and age at the time of treatment [25, 46]. The human oocyte is sensitive to radiation, with an estimated LD50 of $<$2 Gy [47]. This is the lethal dose to destroy 50% of NGF present in the ovary. The number of NGF present at the time of treatment, together with the dose received by the ovaries, will determine the "fertile window" and influence the age of POF. Long-term ovarian failure has been reported in 90% of patients after TBI (10.00–15.75 Gy) and in 97% of females treated with total abdominal irradiation

**Table 2.2** Ovarian failure rates

| Study (year) | Treatment | Age (years) | Ovarian failure (%) |
|---|---|---|---|
| *Breast cancer* | | | |
| Lower (1999) | | Premenopausal | 45 |
| | | <35 | 28 |
| Bines (1996) | | Premenopausal | 68 |
| Meirow (1999) | | <44 | 50 |
| Goodwin (1999) | CMF | 43.7 ± 5.2 | 65 |
| | | <30 | 19 |
| Burstein (2000) | CMF | 30–39 | 30–40 |
| | | <30 | 0 |
| | CAF | 30–39 | 10–25 |
| | AC | <30 | – |
| | | 30–39 | 13 |
| Jonat (2001) | | Premenopausal | 60 |
| *Hodgkin's lymphoma* | | | |
| Howell and Shalet (1998) | Aggressive treatment | | 38–57 |
| Meirow and Dor (2004) | Second-line therapy (not ABVD) | | 32 |
| Bokemeyer (1994) | Infradiaphragmatic Rx | | 50 |
| Brusamolino (2000) | Ovarian-sparing protocol | <25 | 0 |
| | | <45 | 30 |
| Behringer (2005) | Dose-escalated BEACOPP | >30 | 95 |
| | | <30 | 51 |
| *Bone marrow transplantation* | No. of patients | | |
| Sanders (1996) | 73 | 38 (mean) | 99 |
| Teinturier (1998) | 21 | 2–17 | 72 |
| Thibaud (1998) | 31 | 3.2–17 | 80 |
| Meirow (1999) | 63 | 29 (mean) | 79 |
| Grigg (2000) | 19 | 30 (mean) | 100 |
| *Systemic lupus erythematosus* | | | |
| Mok (1998) | 70 | | 26 |
| Boumpas (1993) | 39 (0.5–1.0 g/m$^2$) | | |
| | <7 pulses | | 12 |
| | >15 pulses | | 39 |
| Blumenfeld (1996) | | >30 | 13 |
| | | 20–30 | 15 |
| | | <20 | 100 |
| Appenzeller (2007) | 57, 0.75 mg/m$^2$ | | 17.5 (12.3*) |
| | 50, 0.5 mg/m$^2$ | | 0 (20*) |
| Manger (2006) | 63 patients | <30 | 39 |
| | | 30–40 | 59 |

Adapted and updated from Meirow and Dor [3].
AC, doxorubicin, cyclophosphamide; BEACOPP regimen, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone; CAF, cyclophosphamide, doxorubicin, fluorouracil; CMF, cyclophosphamide, methotrexate, fluorouracil; POF, premature ovarian failure; SLE, systemic lupus erythematosus.

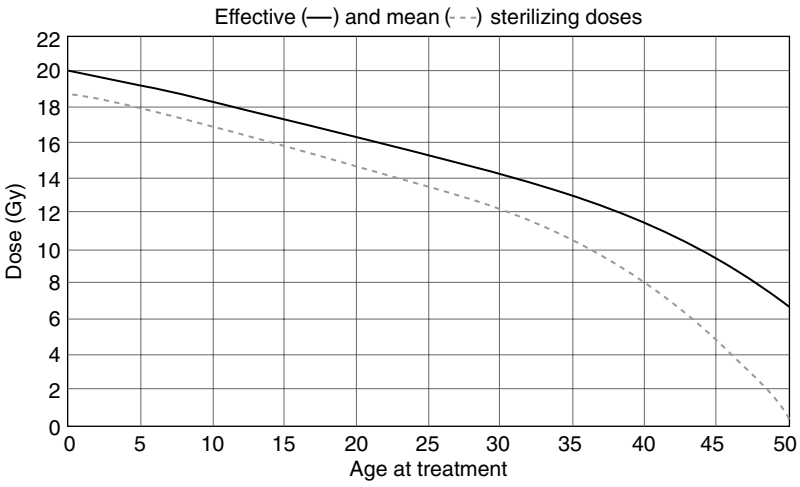


**Figure 2.2** From our understanding of the radiosensitivity of the human oocyte and our knowledge of the natural decline in primordial follicles with increasing age, we can provide effective and mean estimates of the dose required to sterilize a patient at a known age of treatment. Adapted from Wallace *et al.* [51].

**Table 2.3** Risk of permanent amenorrhea in women

| | |
|---|---|
| **High risk** | Stem cell transplantation, external beam irradiation to fields including the ovaries, breast cancer adjuvant combination chemotherapy regimens containing cyclophosphamide, methotrexate, fluorouracil, doxorubicin and epirubicin in women >40 years |
| **Intermediate risk** | Breast cancer adjuvant chemotherapy regimens containing cyclophosphamide in women 30–39 years or doxorubicin/cyclophosphamide in women >40 years |
| **Low risk (< 20%)** | Combination chemotherapy regimens for NHL, ALL or AML breast cancer adjuvant chemotherapy regimens containing cyclophosphamide in women <30 years or doxorubicin/cyclophosphamide in women <40 years |
| **Very low risk or no risk** | Vincristine, methotrexate, fluorouracil |
| **Unknown risk** | Paclitaxel, taxotere, oxaliplatin, irinotecan, trastuzumab, bevacizumab, cetuximab, erlotinib, imatinib |

Adapted from Lee *et al.* [42].
Risk assessment is based on amenorrhea rate. Because some therapies compromise the follicular reserve without causing amenorrhea, fertility may be compromised before the cessation of menses.
ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin's lymphoma.

(20–30 Gy) during childhood [36, 48, 49]. Our understanding of the LD50 of the human oocyte has made it possible to estimate the age at which premature ovarian failure may occur. Furthermore, we have estimated the sterilizing dose following any given dose of radiotherapy at any given age, based upon the application of a mathematical solution to the Faddy–Gosden model for natural oocyte decline (Figure 2.2) [50, 51]. This will help clinicians provide accurate information when counseling women about fertility following treatment for childhood cancer.

Gonadotropin deficiency following high dose cranial irradiation (>24 Gy in the treatment of brain tumors) manifests as delayed puberty or absent menses and can be treated by hormone replacement therapy. Interestingly, early puberty is often reported in females with cranial radiation doses of <24 Gy [52]. However, we have shown a subtle decline in hypothalamic–pituitary–ovarian function following low dose cranial radiotherapy (18–24 Gy). This is characterized by decreased luteinizing hormone (LH) secretion throughout the cycle, an attenuated LH surge and short luteal phases [53], which may compromise reproductive function. Recent data confirm lower fertility in childhood cancer survivors treated with either hypothalamic/pituitary irradiation >30 Gy or ovarian/uterine irradiation >5 Gy [27].

Nowadays, the most commonly used fertility preservation measure is the surgical transposition of the ovaries outside the irradiation field before the initiation of pelvic radiation in adults with gynecological malignancies [54]. This approach may be considered for patients not planning to receive high dose systemic chemotherapy. Surgery is effective at protecting the ovaries from direct irradiation damage, but fertility may be affected by scatter radiation, damage to the

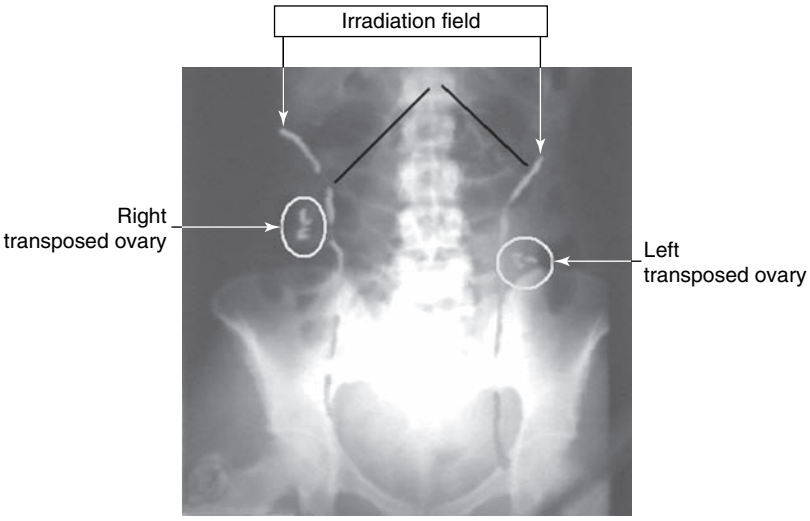


**Figure 2.3** Surgical transposition of the ovaries outside the irradiation field prior to initiation of pelvic radiation for Hodgkin's disease. Adapted from Meirow and Dor [3].

ovarian vasculature during surgery or torsion of the transposed ovary (Figure 2.3). To avoid this, tissue cryopreservation may be considered at the same time as oophoropexy.

## Radiation and the uterus

The uterus is at significant risk of damage following abdominal, pelvic or TBI in a dose and age-dependent manner [55]. Uterine function may be impaired following radiation doses of 14–30 Gy as a consequence of disruption to uterine vasculature and musculature elasticity [48]. Even lower doses of irradiation, as in TBI, have been reported to cause impaired growth and blood flow [56]. The clinical consequences are increased risk of miscarriage and premature delivery [36]. A uterine contribution to an inability to conceive following radiotherapy is not clear but seems likely.

A small number of studies have been reported that attempted to improve uterine function in survivors of cancer with POF. In young adult women previously treated with TBI, physiological sex steroid replacement therapy improves uterine function (blood flow and endometrial thickness) and may potentially allow them to benefit from assisted reproductive technologies [56]. Larsen *et al.* studied uterine volume in 100 childhood cancer survivors and assessed uterine response to high dose estrogen replacement therapy in three patients with ovarian failure and reduced uterine volume following abdominal and/or pelvic irradiation [57]. There was no significant difference in uterine volume, endometrial thickness or uterine artery blood flow following sex steroid treatment, suggesting that higher doses of pelvic radiation cause greater damage than lower doses (as in TBI), and that this damage may be irreversible.

## Testicular function

In males, testicular damage can involve the somatic cells of the testis (Sertoli, peritubular myoid and Leydig cells) or the germ cells. Sertoli cells are responsible for nurturing developing germ cells, and Leydig cells produce testosterone. Recent data have revealed the essential contribution of peritubular myoid cells in mediating the effect of testosterone on spermatogenesis. Gonadal damage in males treated for cancer can result from either systemic chemotherapy or radiotherapy to a field that includes the testes. Cytotoxic treatment targets rapidly dividing cells and it is therefore not surprising that spermatogenesis is impaired after treatment for cancer. The exact mechanism of this damage is uncertain, but it appears to be linked to depletion of the proliferating germ cell pool and associated stem spermatogonial cells. Although the pre-pubertal testis does not complete spermatogenesis and produce mature spermatozoa, cytotoxic treatment given to pre-pubertal boys may impair future fertility. Importantly, the pre-pubertal testis is susceptible to cytotoxic damage.

## Chemotherapy and the testis

As with radiotherapy, the germinal epithelium of the testis is very sensitive to the detrimental effects of

chemotherapy, irrespective of pubertal status at the time of treatment. Therefore, after receiving gonadotoxic agents, patients may be rendered oligospermic or azoospermic. Testosterone production by Leydig cells is usually unaffected, however, and thus secondary sexual characteristics develop normally or are maintained [58]. Following higher cumulative doses of gonadotoxic chemotherapy, Leydig cell dysfunction may also become apparent [59]. Recent data indicate that the proportion of men with mild Leydig cell dysfunction and biochemical, if not clinical, hypogonadism following chemotherapy may be higher than previously recognized [60]. Low testosterone concentrations are associated with a number of important clinical conditions, including osteoporosis, frailty, metabolic syndrome, cardiovascular disease and erectile dysfunction, and thus replacement testosterone should be initiated with appropriate monitoring [61].

Treatment of Hodgkin's lymphoma has involved the use of procarbazine, together with alkylating agents such as chlorambucil, mustine and cyclophosphamide. While these drug combinations have yielded excellent survival rates, the majority of male patients subsequently developed permanent azoospermia [62, 63]. Mackie *et al*. studied patients with a mean age of 12.2 years at diagnosis who were treated with ChlVPP, a regimen containing both chlorambucil and procarbazine [37]. On follow-up, 89% of these patients showed severe damage to the seminiferous epithelium up to 10 years following therapy. Consequently, the treatment of Hodgkin's lymphoma was modified in an attempt to reduce the gonadotoxicity, whilst maintaining long-term survival [64]. Treatment with the ABVD regimen, which contains no alkylating agents or procarbazine, results in significantly less gonadotoxicity, with no patients demonstrating permanent azoospermia [62]. However, anthracycline exposure in this regimen renders it potentially cardiotoxic in the long term. The BEACOPP regimen has high gonadal toxicity in men as in women, with azoospermia reported in 89% of men and low testosterone concentrations in over 50% [65].

## Radiotherapy to the testis

In males, radiation doses as low as 0.1–1.2 Gy can impair spermatogenesis, with doses over 4 Gy causing permanent azoospermia. The somatic cells of the testis are more resistant than the germ cells, and Leydig cell dysfunction is not observed until 20 Gy in pre-pubertal boys and 30 Gy in sexually mature males [66].

Within the pediatric and adolescent age group, testicular damage occurs with direct irradiation to the testes, for example in the management of leukemia [67]. In patients with leukemic infiltration of their testes, radiation doses of 24 Gy are used, and this results in permanent azoospermia [68]. Total body irradiation applied as conditioning treatment before BMT also irradiates the testes. However, the effects of this can be difficult to elucidate as it is usually given concurrently with alkylating agents, but doses of 9–10 Gy have produced gonadal dysfunction [69].

## Chemoprotection

Preventing chemotherapy-induced damage to the ovary or testes remains an elusive ideal in the field of fertility preservation. Most attention has focused on the potential for protecting the ovaries using the gonadotrophin-suppressing gonadotropin-releasing hormone (GnRH) analogues, although a clear biological basis for this approach is unclear as only later stages of follicular growth are gonadotrophin-dependent. There may however be other, indirect mechanisms on smaller follicles, or direct effects of the GnRH analogues themselves, and the approach is supported by animal studies in both rodents [70] and non-human primates [71]. Initial small studies failed to show any benefit [72] but were substantially underpowered, and while later studies showed large apparent benefits [73, 74], lack of randomization and differences between treated and control groups preclude reliable interpretation. A recent randomized controlled trial in women under the age of 40 with breast cancer appears to support a benefit of GnRH analogue treatment [75], but further studies are necessary to substantiate this finding as there are a number of potential confounding methodological issues with study design. Alternative approaches have addressed the mechanisms of chemotherapy-induced oocyte apoptosis using sphingolipids [32] and more recently the c-Abl kinase inhibitor imatinib [76]. In the male, the immunomodulator AS101 has been demonstrated to protect against cyclophosphamide-induced sperm damage and low fertility [77].

## Summary

Whilst many children and adults diagnosed with cancer can now realistically hope for long-term

survival, they must often live with the consequences of their treatment. Infertility is one of the most devastating adverse effects of cancer treatment in this patient group. Both chemotherapy and radiotherapy can impair future fertility, and treatments for certain cancers can be sterilizing [78]. Although predicting individual fertility following treatment is extremely difficult, further epidemiological studies and investigation of markers indicating gonadal damage may be of use to our patients.

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Chapter 3

# Fertility preservation in non-cancer patients

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## Introduction

The increasing survival rates of cancer patients [1–3] have encouraged many specialists to focus on the irreversible consequences of chemotherapy and radiotherapy. Chemotherapy and radiotherapy treatment for cancer or other pathologies has resulted in improved survival rates, but these treatments may also lead to sterility [4]. The increasing success of oncological treatments means it is now even more crucial to implement procedures aimed at preserving fertility.

Similarly to cancer patients, there are some non-oncological conditions currently treated with gonadotoxic agents, such as patients with autoimmune disorders or some chromosomal abnormalities that can lead to ovarian failure. There are also other situations where a woman may benefit from fertility preservation procedures, such as a woman with severe or recurrent endometriosis or a woman who wants to postpone conception until her late reproductive years [5].

In addition, there are other gynecological situations regularly found in our clinical practice that were previously difficult to deal with but which now can be solved. Thus, oocyte or embryo vitrification can be performed when, for any reason, we should prefer to transfer embryos in a different cycle to the stimulated one. People with a high risk of hyperstimulation syndrome, the presence of a hydrosalpinx or polyps during the stimulation, the absence of sperm in the sample the day of the ovum pick-up or any bleeding previously to embryo transfer are some situations where vitrification should be considered. As a large number of embryos are needed, vitrification may also be helpful for the low-responder patient with the aim of accumulating oocytes or embryos, especially if pre-implantation genetic screening (PGS) is planned [6].

Fertility preservation is already well established in males. It may simply be solved by freezing sperm samples [7], and should be considered in all cases a specialist may suspect any prospective damage to sperm or to testicular function. More recently, it has become possible to preserve young females' gonadal function and fertility. In this chapter we will focus on female fertility preservation procedures because of their complexity and peculiarities.

Ovarian failure leads to the impossibility of childbearing apart from other problems related to the menopause, such as vasomotor, skeletal or cardiovascular alterations. Early menopause and infertility are two of the main consequences for patients treated with gonadotoxic agents. Interest in fertility preservation has grown due to the effect of infertility on a woman's quality of life and self-esteem.

Lately, oocyte vitrification and ovarian tissue cryopreservation procedures have been modified with excellent clinical outcomes [8, 9]. But these are not the only methods with which to preserve fertility. The use of gonadotropin-releasing hormone agonists (GnRHa) is also of interest. In addition, although it is still considered experimental, immature oocyte retrieval for the in vitro maturation (IVM) process provides great expectations for future fertility preservation [10, 11].

Since fertility preservation procedures show a benefit for patients to be treated with gonadotoxic agents, a new field in assisted reproductive technology (ART) has appeared. As users of testicular and ovarian preservation techniques, patients undergoing treatment with gonadotoxic agents are a new population in fertility clinics. However, fertility preservation procedures should not be limited to patients undergoing cancer therapy but also applied to any situation where

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reproductive function is threatened. If fertility may be diminished, specialists, patients and their families should be aware that fertility preservation is an option and that reproductive function may be preserved.

Although some fertility preservation methods need to be improved, they still must be disseminated among medical professionals and patients with the aim of preventing infertility. Future fertility or a pregnancy is not guaranteed by fertility preservation procedures but, if done, these procedures may help people face any treatment with a high risk of ovarian failure, offering them future hope [12].

## Ovarian damage and decreased activity

Gonadotoxicity – a decrease in ovarian activity – depends on several factors, including the age of the patient; the initial status of the ovaries (referred to as the antral follicular account); the treatment applied (chemotherapy, radiotherapy or surgery) and cumulative doses; and the type of agent used.

As many factors may contribute, it is difficult to establish the exact incidence of premature ovarian failure after systemic chemotherapy. Most ovarian failure data are referenced to cancer patients, who receive higher doses of chemotherapy and, consequently, have an increased incidence of ovarian failure. People with autoimmune disorders may also be treated with chemotherapy but usually at lower doses than cancer patients. So, chemotherapy's consequences may not be so dreadful but determined by the cumulative dose.

Although many of the patients treated with chemotherapy recover their ovarian function once chemotherapy is completed, there is an increased risk of premature ovarian failure, especially when related to age [13] and the use of alkylating agents [14, 15].

### Age

The ovarian cortex has a limited number of primordial follicles that decrease with age. Previous chemotherapy or surgery can also affect the initial status of gonads before treatment starts. This will determine the final impact on ovarian function.

Cumulative doses of cyclophosphamide can cause infertility in young women [16]. Gonadotoxicity is directly related to age: the cumulative dose needed to cause premature ovarian failure decreases as age increases. Its effect can be acute or cumulative, and the ovarian capacity for recovering is limited. Chemotherapy and radiotherapy frequently induce a reduction in the number of germ cells, with a loss of steroid hormones, the possibility of mutation or teratogenic consequences [17].

The implications of chemotherapy treatment on fertility and future pregnancy has a higher relevance for younger women as most are childless or haven't completed their family. But, as gonadotoxicity is an age-related process, their younger age will have a protective effect. Many younger patients will naturally recover their ovarian function and fertility, especially if the applied chemotherapy doses are low.

### Chemotherapy

It is known that chemotherapeutic agents can cause mutations, DNA adducts and structural breaks, as well as oxidative damage in somatic and germ cells. Alkylating agents such as cyclophosphamide or ifosfamide are the most gonadotoxic agents, but also gonadotoxic are chlorambucil, busulfan, cisplatin, melphalan, carboplatin or procarbazine [14, 15, 18].

The effect of chemotherapy on the ovary is not an all-or-nothing phenomenon, so the number of surviving primordial follicles following chemotherapy will depend on several factors such as age, type of agent and doses received [16].

Chemotherapy's alkylating agents join with DNA, avoiding its replication and transcription [18]. They are extremely gonadotoxic by acting at any phase of the cellular cycle (cellular cycle phase independent), causing damage to the primary follicles. Pathological examinations of ovarian biopsies in patients treated with cyclophosphamide show either a total absence or a significant reduction in the number of inactive follicles, with fibrosis and no signs of follicular maturation [19].

The mechanism of chemotherapy causing premature ovarian failure is not well known, but granulosa cells appear to be the crucially affected cells [20]. Cellular edema of pre-granulosa cells is observed, with queratin deposits and edema of the nucleus of the cell, which damages the oocyte morphology [21]. Additional factors, such as vascular alterations and fibrosis of the ovarian cortex, may contribute to the reduction of follicles [22].

What is clear is that both the antral follicle count and ovarian volume decrease after chemotherapy.

A fast fall in anti-Müllerian hormone (AMH) and inhibin B concentrations is observed during chemotherapy, although estradiol concentrations are maintained [23].

### Radiotherapy

Similar to chemotherapy, the effect of radiotherapy on the gonads depends on age, cumulative doses, fractioned doses and irradiation area. The average doses needed to destroy oocytes in humans is <2 Gy [24]. Ninety-seven percent of women receiving 5.0–10.5 Gy will subsequently undergo ovarian failure [25].

Especially if radiotherapy is applied during childhood, the irradiation area has been associated with alterations of the uterine function due to the reduction of vascular flow and endometrial thickness [26]. Cranial irradiation with 35–45 Gy doses can damage the hypothalamus–pituitary–gonadal axis but, as gonads are not affected, they recover their function with gonadotropin replacement.

### Surgery

Repeat ovarian surgery due to endometriosis or another benign pathology may diminish the ovarian reserve and lead to premature ovarian failure. Furthermore, in recurrent endometriosis, normal residual ovarian tissue may be compromised.

Tubal sterilization through electrocoagulation, when compared to the application of mechanical clips, also seems to have an adverse effect on ovarian reserve in the postoperative period. Significant differences have been detected in ovarian volumes at day 3 and antral follicle counts at 10 months after the tubal occlusion [27].

### Treatment for rheumatic diseases

There are four main categories of drugs for the treatment of rheumatic diseases: (1) anti-inflammatory drugs; (2) corticosteroids; (3) immunosuppressive drugs; and (4) biological agents. These treatments are split into two main groups: the disease modifying anti-rheumatic drugs (DMARDs) and the non-steroidal anti-inflammatory drugs (NSAIDs).

### Disease modifying anti-rheumatic drugs

Disease modifying anti-rheumatic drugs are a host of new drugs. Although most of the patients diagnosed with rheumatic diseases are treated with non-biological DMARDs, the rate of biological DMARDs is increasing. The gonadotoxic effects of the anti-inflammatory and immunosuppressive drugs have not been studied with the exception of salazopyrine and some cytotoxic drugs as described below.

- ***Salazopyrine*** impairs fertility in males, although not females, with a higher incidence of oligospermia, decreased sperm motility and higher rates of abnormal forms. Men with inflammatory bowel disease treated with salazopyrine showed a higher incidence of fetal abnormalities among offspring. Folate deficiency may have a role, as salazopyrine inhibits the gastrointestinal and cellular uptake of folate [28], but salazopyrine also has its own role as fetal abnormalities weren't avoided with folate supplementation. An oxidative stress mechanism of male-induced infertility has also been described [29]. Usually, spermatogenesis recovers at about 2 months after withdrawal of the drug [30].
- ***Cyclophosphamide and chlorambucil*** are rarely used in the treatment of rheumatoid arthritis, but these drugs are very important for patients with systemic lupus erythematosus. Cyclophosphamide is gonadotoxic in both sexes. It is not possible to predict which patients will become infertile and which will recover reproductive function, this depending fundamentally on age and the cumulative dose [30].

### Non-steroidal anti-inflammatory drugs

Inhibitors of cyclooxygenases (COX-1 and COX-2) are involved in ovulation and implantation. Transient infertility has been described after treatment with NSAIDs, such as indomethacin, diclofenac, piroxicam and naproxen. Non-steroidal anti-inflammatory drugs can inhibit the rupture of the luteinized follicle and, thereby, cause transient infertility [30].

A decreased sperm count has been found in chronic male users of NSAIDs at low or moderate doses [31].

## Fertility preservation procedures

Several strategies have been proposed over the last few years to protect and preserve the ovarian function in patients with cancer or suffering from other pathologies with a high risk of premature ovarian failure. Some

have demonstrated their efficiency and are now part of the daily routine of clinical practice, while others are still under evaluation.

These options include embryo and oocyte cryopreservation, cortical or whole ovary cryopreservation and GnRHa protection. In vitro maturation of imature oocytes still needs improvement, but there is no doubt that it will become an important part of these procedures in the future as the trend in fertility preservation techniques is directed towards ovarian tissue cryopreservation and further retrieval of immature oocytes followed by IVM and vitrification [32].

Unlike with cancer patients for whom chemotherapy needs to be started immediately, other patients usually have no problem with the time frame of the 2–3 weeks needed to obtain the oocytes, as there is no hurry to complete the ovarian stimulation. Neither patients with endometriosis nor young people who wish to postpone childbearing are inconvenienced by this time frame. Indeed, some stimulations cycles can even be performed to increase the number of oocytes when oocyte vitrification is intended.

## Oocyte vitrification

Oocyte vitrification is a method of cryopreserving human oocytes which provides an excellent clinical outcome [33]. Vitrification is solidification of a solution by an extreme elevation of viscosity using high cooling rates, from −15 000 to −30 000°C/min, which avoids ice crystal formation and, thus, the damage and the osmotic effects caused by intracellular ice formation. One of the problems of vitrification is the toxicity of cryoprotectants. This can be reduced by the use of an adequate combination of cryoprotectants (ethylene glycol + dimethylsulfoxide [DMSO] + sucrose) or by using very low volumes, which increases the speed of the vitrification process and consequently reduces the use of cryoprotectant in the vitrification solution [34].

The *Cryotop method* is a minimal volume device where oocytes are vitrified in volumes <0.1 μl, which preserves their capacity for fertilization and further development after warming. Survival rates of 97% have been referred, with no differences in fecundation and implantation rates, embryo quality or pregnancy rates when compared to fresh oocytes [35].

Historically, the slow cooling method for oocyte cryopreservation has been hampered by its low efficiency and because it did not guarantee reproducible results. However, recently the success rate has increased due to a better understanding of oocyte physiology, the use of improved media and the implementation of new techniques [35]. The number of pregnancies resulting from oocyte cryopreservation is constantly increasing, with no apparent increase in adverse postnatal outcome such as low birth weight or congenital abnormalities [36].

## Embryo cryopreservation

Embryo cryopreservation is a widely accepted method that is now considered the standard practice for fertility preservation [37]. Previously, oocytes had to be fertilized to be preserved, as thawed embryos were considered to achieve higher survival rates than oocytes. The female patient needed to have a partner or sperm donor to fertilize the retrieved oocytes, creating embryos that may not have been used in the future, which had various ethical considerations. Oocyte cryopreservation avoids the need for sperm at the time of oocyte retrieval and the results have been similar to embryo cryopreservation, and thus should be considered an option.

## Ovarian tissue cryopreservation

Ovarian tissue freezing for later autotransplantation is another alternative for fertility preservation in women with oncological or non-oncological diseases [38]. Immature oocytes in primordial follicles of the ovarian cortex are less sensitive to cryopreservation damage [39]. Thus, ovarian tissue freezing is an alternative to ovarian stimulation and oocyte cryopreservation for preserving fertility. Orthotopic transplantation of the frozen–thawed ovarian cortex would allow natural fertility and, in the case of failure, in vitro fertilization would still remain an option. Another advantage of this approach, apart from future childbearing, is that patients would be able to restore ovarian function. Ovarian cryopreservation and transplantation procedures have so far been almost exclusively limited to avascular cortical fragments. Transplantation of an intact ovary with vascular anastomosis has been proposed as a way to reduce the ischemic interval between transplantation and revascularization. To date, there are only a few newborns from this technique [40]. Recently, 2 newborns, one miscarriage at 7 weeks and a biochemical pregnancy were related following 6 reimplants [41]. This publication was interesting because in all 6 patients the whole ovary was cryopreserved prior to treatment, and tissue from 3 of the patients was

transported 4–5 h on ice prior to freezing, demonstrating that hospitals may offer cryopreservation without having the necessary local infrastructure.

The main disadvantage of this technique is that it requires surgery – laparoscopy – to obtain the ovarian tissue and a further re-implant with appropriate incorporation of the cryoprotectant to the tissue. Ischemic damage and reduced follicular pool usually appear after transplantation. The active life of the transplanted tissue will depend on the neoangiogenesis and new vascularization.

This approach offers great possibilities to patients in the future, since portions of healthy tissue can be preserved for a further use when an oophorectomy is performed for a benign indication. The ethical basis for performing this surgery for elective cryopreservation has been discussed [42, 43], but a patient's request for cryopreserving small portions of ovary at the time of any other gynecologic surgery should not be denied on ethical grounds.

Currently, ovarian tissue cryopreservation is only recommended as an experimental treatment in selected patients. Research is needed to investigate the revascularization process with the aim of reducing the follicular loss that occurs after tissue grafting.

Ovarian tissue cryopreservation can serve as a source of follicle for IVM. Thus, though still experimental, future fertility preservation techniques will tend towards combining ovarian cryopreservation and immature egg retrieval for further in-vitro oocyte maturation and vitrification. To become widely accepted, these procedures need to be safe, easy to perform and with favorable results. Meanwhile, a combination of ovarian cryopreserved transplantation, further ovarian stimulation and vitrification of the retrieved oocytes to be accumulated for an in vitro fertilization (IVF) cycle can be used. A twin pregnancy was recently obtained through this method [44].

## Gonadal medical protection

### Gonadotropin-releasing hormone agonists

Although there is controversy about the use of GnRHa preventing the ovaries from being damaged after chemotherapy, the latest prospective randomized studies do show a benefit [18, 48, 49]. Following the administration of GnRHa, a reduction in the mitotic activity of the granulosa cells has been described [50]. In some studies performed on mice treated with GnRHa, inhibition of the recruitment process of preantral follicles and their evolution to antral follicles was demonstrated. Thus, GnRHa could avoid follicles reaching their sensitivity threshold to chemotherapy by supression of the granulosa cells. But it is not yet known if the effect would be similar in humans, as the GnRHa protective effect would not be sufficient enough for the regimens used in humans, which are usually longer and with higher doses of chemotherapy than those protocols used in animals. A reduction in the response of breast cancer to chemotherapy can be observed, which is due to the GnRHa effect blocking the cells in G0 phase of the cellular cycle. This causes cells to become resistant to chemotherapy as it is considered that 50% of tumoral cells in breast cancer have receptors for GnRH and its analogues [51]. Experimental studies have demonstrated that both GnRH agonists and antagonists directly inhibit ovarian cancer proliferation through GnRH receptors overexpressed on 80% of these tumors [49].

Patients with cyclophosphamide-treated systemic lupus erythematosus or other autoimmune diseases urgently need ovarian protection as, although doses of chemotherapy are usually not as high as those given for cancer treatments, there is a high premature ovarian failure rate [52]. Because of this, GnRHa protection should be considered, as well as other ART fertility preservation procedures.

### Imatinib

Imatinib acts by blocking the apoptotic pathway activated by cisplatin in ovarian germ cells. Cisplatin induces DNA damage by activating the c-Abl–TAp63 pathway, leading to cell death. In cell lines, c-Abl phosphorylates TAp63, which induces the activation of proapoptotic cells. Treatment with the c-Abl kinase inhibitor imatinib blocks these effects [53].

## In-vitro maturation

Although of great interest for the future, immature egg retrieval for further in-vitro oocyte maturation and vitrification is currently not a feasible option.

Most of the follicles in human ovaries remain primordial. Thus, they would be the most abundant source of oocytes but, due to their immaturity, IVM is needed. Primordial follicles can be isolated from either fresh or cryopreserved ovarian tissues and matured in vitro for further vitrification.

Many healthy children with normal outcomes have been born after using this method [54]. The main

benefit is the absence of stimulation and its low cost, but results are not consistent enough and still need to be improved [11, 54]. Pregnancy and implantation rates are lower than those obtained with standard IVF cycles [55], and a higher clinical miscarriage rate has been observed [56]. So, more controlled studies are needed of the possible long-term effects of IVM on babies.

This method may be considered for patients in whom hormonal ovarian stimulation is not recommended due to high estradiol levels, such as breast cancer patients or those suffering from systemic lupus erythematosus. It may also be suitable for patients with polycystic ovary syndrome (PCOS) or when there is an urgent need to start cytotoxic therapy.

### Transposition of ovaries

Scatter radiotherapy can cause considerable damage even if the gonads are outside the radiation field. The purpose of this approach is to avoid the direct exposure of the ovaries to radiotherapy, although the indirect exposure can also cause gonadotoxicity. Thus, it should be indicated for any pathology that requires pelvic radiotherapy treatment. When this approach is performed, 16–90% of the patients show the ovarian function preserved [45–47].

Ovarian transposition is not suitable for non-oncological patients as radiotherapy is uncommonly used, although it is very useful for cancer patients when they are going to receive local radiotherapy.

## Indications for fertility preservation in non-oncological patients

Fertility preservation is not limited to cancer patients, and can be considered and offered to patients with many other ordinary conditions. Any patient with a high risk of premature ovarian failure is a possible candidate for fertility preservation. The indications include those listed in Tables 3.1 and 3.2.

### Autoimmune diseases

The effect of autoimmune disorders on fertility depends most of all on a woman's reproductive age. Fertility preservation methods should be considered in young women with severe systemic autoimmune rheumatic diseases requiring imminent gonadotoxic treatment.

**Table 3.1** Ovarian tissue cryopreservation: the Valencia program for fertility preservation

| **Malignant** | **(*n* = 284)** |
|---|---|
| Breast cancer | 170 |
| Hodgkin's lymphoma | 62 |
| Other tumors | 52 |
| **Non-malignant** | **(*n* = 17)** |
| Systemic lupus erythematosus | 3 |
| Crohn disease | 1 |
| Endometriosis | 5 |
| Nephropathies | 5 |
| Multiple sclerosis | 2 |
| Wegener's granulomatosis | 1 |

The availability of new medications for the treatment of rheumatoid arthritis has dramatically changed the prognosis for these patients, but limited data about their reproductive or developmental toxicity are available, particularly when the medications are used for the treatment of rheumatoid arthritis.

Cyclophosphamide is only used for refractory rheumatoid arthritis. It seems to have a significant beneficial effect on the clinical evolution of rheumatoid arthritis, but its administration has been limited due to its secondary aggressive effects and the similar benefits and low risk of other anti-rheumatic agents. Furthermore, cyclophosphamide treatment for rheumatoid arthritis has been related to the appearance of some hematological malignant neoplasms and bladder cancer [57, 58].

Cyclophosphamide is the elective drug of choice in the treatment of severe manifestations of systemic lupus erythematosus, such as proliferative nephritis, affection of the central nervous system, pneumonitis or severe thromobocytopenia [59].

Cyclophosphamide-treated lupus patients have premature ovarian failure rates of up to 50% in women below 30 years and 60% in women between 30 and 40 years of age [60]. In a manner similar to onocological patients, as survival rates have increased ovarian failure and infertility have become important issues for these patients.

The use of intermittent cyclophosphamide has been proposed due to its successful application in systemic lupus erythematosus and the observed reduction in toxicity. However, there is continued controversy over its results, and it remains unclear if secondary effects of cyclophosphamide are influenced

**Table 3.2** Oocyte vitrification with non-oncological indication: Instituto Valenciano de Infertilidad (IVI), Valencia, Spain

| | No. patients | Age | No. vitrified oocytes |
|---|---|---|---|
| Autoimmune diseases | 2 | 33 ± 3.2 | 20 (10 ± 4) |
| Postponing childbearing | 65 | 34 ± 6.1 | 890 (7.7 ± 5.3) |
| Gynecological disorders in ART (bleeding, hydrosalpinx, hydrometra) | 67 | 35.8 ± 4.2 | 532 (7.9 ± 5.6) |
| Gynecological disorders non-ART | 65 | 36 ± 4.2 | 346 (5.8 ± 4.8) |
| Lack of partner's sperm sample | 18 | 35 ± 4.1 | 184 (10.8 ± 5.5) |
| OHSS | 12 | 32 ± 4.3 | 286 (19.0 ± 4.5) |
| Accumulation previous to PGS-FISH | 348 | 39.5 ± 3.5 | 2050 (2.5 ± 2.6) |
| Accumulation previous to PGS-PCR | 28 | 35.5 ± 3.3 | 264 (5.1 ± 3.3) |
| Low response | 543 | 36.7 ± 3.2 | 2163 (2.6 ± 1.6) |

ART, assisted reproductive technology; PGS, pre-implantation genetic screening; FISH, fluorescence in-situ hybridization; PCR, polymerase chain reaction; OHSS, ovarian hyperstimulation syndrome.

by the frequency of administration or the cumulative dose taken by a patient [58].

There is a concern that exogenous female hormones may worsen disease activity in women with systemic lupus erythematosus. Due to this, rheumatologists have traditionally discouraged the use of estrogens in lupus patients. Recent investigations have shown that estrogens can cause light cutaneous eruptions, but do not decrease disease activity. But these findings cannot be applied to women with high levels of anti-cardiolipin antibodies, lupus anticoagulant or previous thrombosis with low activity lupus, in whom the use of estrogens may increase the risk of severe lupus crises [61].

Estrogen may have some use when oocyte cryopreservation is desired, because ovarian stimulation is needed. Thus, high estradiol levels can result in patients with severe lupus being stimulated with gonadotrophins. In these cases, letrozole can be used for the stimulation in a manner similar to estrogen sensitive cancers. If not, IVM could be an option.

Letrozole is an aromatase inhibitor that has been shown to be effective as an ovulation inductor because it reduces the negative feedback that estradiol exerts in the hypothalamus and pituitary. This results in an increased follicle stimulating hormone (FSH) sensitivity in ovarian granulosa cell receptors. Furthermore, letrozole induces suppression of circulating estradiol levels [62]. It can be used alone or in combination with FSH. Letrozole with FSH results in a higher number of oocytes that can be vitrified with lower estradiol levels, in a manner similar to physiologicals [63].

As described by Oktay, letrozole stimulation consists of oral administration of 5 mg of letrozole beginning on the 2nd or 3rd day of the cycle and continuing until the day of human chorionic gonadotropin (hCG) administration. After 2 days of letrozole administration, 150 U of gonadotropins are added. A GnRH antagonist is administered when a follicle reaches 14–15 mm or the E2 level exceeds 250 pg/ml, and hCG is given when the leader follicles are >20 mm. Letrozole administration is continued until the appearance of menses [63].

## Postponement of childbearing (age)

Social and economic factors associated with modern lifestyles have resulted in many women choosing to delay having children, and so the number of infertile couples has risen over the last few years. Population data reveal an increase the age at which both men and women attempt to conceive their first child.

Over the past 10 years the mean age at which a woman conceived her first child rose by more than 2 years. The negative effect of age on fertility is well established, especially for women over 35 years of age for whom poorer quality oocytes and decreasing ovarian reserve are of greater concern that with younger women [47, 64]. Reproductive trends and the physiological factors associated with conception in older women point to growing problems with infertility in the future.

Cryobanking their own healthy "young" oocytes is a good option for women who plan to conceive children late in their reproductive life. This option is even

more attractive for women who have a family history of premature menopause or factors that predispose them to premature ovarian failure.

## Recurrent ovarian surgery (endometriosis)

Repetitive surgery on the ovary due to endometriosis or any other benign pathology can diminish ovarian reserve and lead to premature ovarian failure.

Endometriosis is one of the most frequent pathologies in gynecological surgery. Although there is no evidence that surgery can cure infertility, cyst excision is considered one of the best options to avoid recurrence of endometriosis and improve fertility. However, excision of endometriotic cysts is also associated with a significant reduction in ovarian reserve [65], and so electrosurgical coagulation plays an important role. Ovarian damage and premature ovarian failure incidence may increase in the case of bilateral endometriotic cysts.

Endometriomas should be removed only in case of pain, infertility or if it is an asymptomatic endometrioma over 4 cm in size. It is still unclear which is the best approach when assisted reproduction techniques are used, but convincing evidence has emerged showing that responsiveness to gonadotrophins is reduced after ovarian cystectomy and there are no deleterious effect of surgery on pregnancy rates. So, proceeding directly to IVF is recommended to reduce the time to pregnancy and to avoid potential surgical complications. Surgery should be considered only for large cysts and smaller ones that can obstruct the ovum pick-up because of their location in the ovary [66].

Excessive surgery often leads to destruction of normal ovarian tissue, which is usually excised along with the endometrioma wall. Analysis of excised tissue reveals that most of the follicies were close to the ovarian hilus. Incomplete surgery is associated with an increased risk of recurrence [67], but there is no clear evidence as to whether the damage is related to the surgical procedure, to the previous presence of endometriomas or both [68]. Following surgery, an important reduction of the ovarian response to stimulation is observed, but this does not seem to be related to the dimension of the excised ovarian cyst [65].

In patients with a high risk of ovarian failure, oocyte vitrification or preservation of ovarian tissue should be considered before surgery. When a cystectomy or an oophorectomy is performed due to a benign indication, portions of healthy tissue could be preserved for future use.

## Bone marrow transplantation

Bone marrow transplantation for the treatment of both oncological and non-oncological hematological diseases leads to ovarian failure due to the aggressive chemotherapy and radiotherapy used to destroy preexisting bone marrow [69].

## Chromosomal abnormalities that can lead to premature ovarian failure: Turner's syndrome

Premature ovarian failure is a common condition of Turner's syndrome. Fertility preservation may not be feasible for most patients with Turner's syndrome. After careful consideration of increased pregnancy associated risks, it can be recommended for young age patients with mosaic Turner's syndrome [70].

Spontaneous puberty occurs in 20–30% of Turner's syndrome patients and their fertility rates are about 5–10% [71]. This indicates the presence and maturation of follicles when young, and so this is probably the best time to attempt fertility preservation procedures.

## Ovarian borderline tumors

An ovarian borderline tumor is an epithelial tumor with a low potential to invade or metastasize. This low malignant potential tumor accounts for 10–15% of ovarian epithelial tumors. Nearly 80% are stage I at the time of diagnosis. Prognosis will depend on the histological type and stage at surgery, serous and mucinous being the most frequent histological types. The 5-year survival rate for women with stage-I borderline tumors is about 95–97%, but the 10-year survival rate is only between 70 and 95% because of late recurrence. The survival rate for advanced stage serous borderline tumors with non-invasive implants is 85%, while the rate for tumors with invasive implants is 60%. Mucinous borderline tumors are usually gastrointestinal. Survival rates are good, except when they are associated with peritoneal pseudomyxoma, which has bad prognosis due to its extraovarian origin [72].

Treatment for borderline ovarian tumors is similar to that for ovarian cancer and includes hysterectomy with bilateral adnexectomy. However, patients with borderline ovarian tumors tend to be younger

than women with invasive ovarian cancer, with many of them nuliparous, and so conservative surgery (cystectomy or unilateral oophorectomy) must be considered for patients with early stage borderline ovarian tumors [73]. Lately, this concern has been expanded to women with advanced stage disease [74]. Conservative management increases the risk of recurrence but does not affect survival. Recurrence rates are higher when a cystectomy is performed (58%) instead of an adnexectomy (23%) [73]. Following this treatment, fertility, pregnancy outcome and survival rates remain excellent.

A biopsy must be performed when any macroscopic implant can be observed on the contralateral ovarian surface. Thirty percent of serous borderline tumors are bilateral and, because these tumors often are diagnosed after surgery, fertility preservation should be recommended before the procedure. If ovarian preservation is impossible, oocyte or ovarian cryopreservation before the surgical procedure must be considered [74].

In any case, fertility preservation can also be done as preventative measure in case of recurrence and the need for an adnexectomy.

Similar to borderline ovarian tumors, patient carriers of the BRCA-1 or BRCA-2 mutations have an increased risk of breast and ovarian cancer, and so a patient may undergo adnexectomy due to cancer or just as prevention. Fertility preservation techniques may also be recommended in such circumstances.

## Other routine gynecological situations

There are some other clinical gynecological situations in the routine clinical practice of ART that can be solved by using cryopreserved oocytes. These situations are as follows:

- Patients with a high risk of ovarian hyperstimulation syndrome to whom embryo transfer would not be desirable.
- To accumulate oocytes in low-responder patients, for a further IVF or pre-implantation genetic screening (PGS) cycle [75].
- To postpone transfer to another cycle due to finding a hydrosalpinx or polyp, or any bleeding or hydrometra that may appear previous to transfer [75].
- Semen or pathological samples are not available the day of the oocyte retrieval.
- Inappropriate endometrium for transfer [75].
- Establishment of egg-banking for ovum donation programs. This would simplify the donation process, since no synchronization between donor and recipient is needed. Oocyte cryopreservation would also ensure safer oocyte donations in a manner similar to semen banks because it would allow more accurate screening and quarantine for viral infections.

# Ethical concerns about embryo freezing

Previously oocytes needed to be fertilized to be preserved because embryo thawing was thought to achieve higher survival rates than oocytes cryopreservation. Oocytes were fertilized with sperm samples from the patient's partner or a sperm donor, with different ethical considerations for each.

Oocyte and ovarian tissue cryopreservation are useful as they overcome some of the disadvantages, ethical concerns and legal restrictions related to embryo cryopreservation.

# Conclusions

For fertility clinics, advances in fertility preservation procedures have created a new group of patients who seek to use testicular and ovarian preservation techniques in order to prevent infertility. Specialists should inform patients about the new approaches that will allow them to attempt a pregnancy in the future with their own oocytes, regardless of whether they experience ovarian failure.

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Chapter 4

# Basic cancer biology and immunology

Roy A. Jensen, Lisa M. Harlan-Williams, Wenjia Wang and Shane R. Stecklein

## Introduction

Cancer describes a group of well over 200 diseases, each with distinct and heterogeneous molecular aberrations that result in a breakdown of cellular mechanisms that govern cell growth, death and differentiation. From the early observation of chromosomal abnormalities in cancer cells, then the identification of the first proto-oncogene, and now the detailed analyses of specific genes and signaling networks, we have come to recognize cancer as an astoundingly complex acquired genetic disease.

## The origin of cancer

### Transformation of normal cells

In its infancy, the science of histopathology revealed that like normal tissues, tumors are composed of cells. This rudimentary observation proved fundamental in our understanding that tumors were not the manifestation of some external entity, but rather were aberrant progeny of our own cells. More detailed analyses coupled with improvements in technology allowed for the categorization of tumors with respect to their tissue and cell of origin and the microscopic behavior of the mass. Malignancies which arise from epithelial, mesenchymal and primitive tissues were termed ***carcinomas***, ***sarcomas*** and ***blastomas***, respectively. Furthermore, those neoplasms that retained some semblance of the normal tissue and grew in a confined manner were termed ***benign***, while those that grew and invaded the surrounding tissue and/or gave rise to distant metastases were termed ***malignant***. Today, the cellular origin of a diverse array of human cancers is well-known. The remainder of this chapter discusses our current understanding of the fundamental molecular abnormalities that contribute to malignant transformation of normal cells, the role of the normal and cancer-associated immune system and the biological manifestations of cancer.

### Oncogenes and tumor suppressor genes

In the 1970s, tumor viruses were believed to be the cause of human cancers and efforts were made to identify the mechanism(s) by which these viruses could redirect the cellular machinery of their host to proliferate uncontrollably. However, attempts to isolate these viruses from human tumors proved unsuccessful, which resulted in a paradigm shift towards the role of carcinogens as mutagens that mutate normal cellular genes, or ***proto-oncogenes***, to ***oncogenes*** [1]. This was made possible using a novel experimental procedure of transfection to demonstrate that DNA from chemically transformed cells could induce cellular transformation in recipient cells, suggesting that the donor cells carried genes that could function as oncogenes [2]. Mechanisms that have been implicated for oncogene activation include mutation (e.g. point mutation in H-*ras*), gene amplification (e.g. *eRB1B2/neu/HER2*) and chromosomal translocation (e.g. Bcr-Abl) [3].

The technique of cell fusion between a normal cell and a cancer cell derived from a non-virus-induced tumor often resulted in non-tumorigenic hybrid cells, suggesting that genes from the normal cell can substitute for those in the cancer cell and that these genes antagonize the cancer cell phenotype [4]. But this approach did not identify specific ***tumor suppressor genes***. Studies involving the *RB1* gene provided a genetic explanation. Alfred Knudson Jr. postulated from the kinetics of the appearance of unilateral (sporadic) and bilateral (familial) retinoblastomas that the familial form, having already inherited one *RB1* gene mutation, only needs to sustain a single somatic

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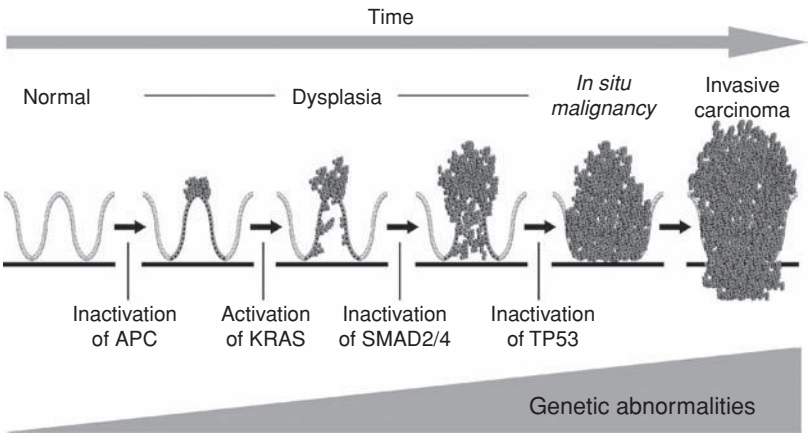


**Figure 4.1** Overview of the stepwise accumulation of mutations in colorectal cancer.

mutation whereas the sporadic form requires two somatic mutations [5]. The inactivation of the two copies of a tumor suppressor gene can occur by mutation, by loss of heterozygosity due to mitotic recombination or gene conversion or by promoter methylation [6, 7].

### Stepwise accumulation of mutations

The identification and early functional characterization of oncogenes and tumor suppressor genes illuminated the role of genetic mutation in the pathogenesis of cancer. While mutations in these genes set the stage for our understanding of tumorigenesis, mounting evidence suggested that inactivating a tumor suppressor gene or promoting the activity of an oncogene alone is not sufficient to induce malignant behavior. We now know that a series of mutations in the lifetime of a cell are required in order to overcome the superbly effective regulatory mechanisms intrinsic to a cell that regulate orderly cellular division, differentiation and death. While there is evidence supporting this stepwise accumulation of mutations in the vast majority of human malignancies, this process was first elegantly demonstrated in the case of colorectal cancer [8].

Mutational inactivation of the adenomatous polyposis coli (*APC*) tumor suppressor gene is observed in approximately 85% of all early stage adenomatous polyps and dysplastic crypt foci, which are considered to be premalignant neoplasms [9]. This suggests that loss of *APC* is among the earliest genetic lesions in the progression of colorectal cancer. However, additional sequential mutations have been identified in *KRAS*, *SMAD2/4* and *TP53* and are thought to be critical for the progression of premalignant polyp to invasive adenocarcinoma (Figure 4.1) [8, 9].

### The "cancer stem cell" theory of cancer

Despite our growing knowledge of the molecular events that lead to malignancy, a number of fundamental questions remain concerning the cellular etiology of cancer. A growing body of evidence suggests that only a small number of cells within most human cancers truly possess tumorigenic properties. This was first demonstrated definitively in human acute myelogenous leukemia, where transplantation of a single human leukemic cell into an immunocompromised mouse was capable of recapitulating the phenotypic heterogeneity and malignant features observed in the human patient [10]. Thus the paradigm of a ***cancer stem cell*** (CSC) was born. Accumulating evidence now supports the existence of CSCs in a number of solid cancers, including those of the breast, brain, colon and prostate [11–15].

Tissues which undergo regeneration, remodeling and renewal during an organism's lifetime are thought to possess a small population of quiescent stem cells which respond to injury and microenvironmental cues in order to maintain tissue homeostasis. It is this long-lived population of cells, seated at the apex of a hierarchical differentiation pathway that establishes the various cellular components of adult tissues through asymmetric division (Figure 4.2). Given that most human epithelial cancers arise in tissues with relatively rapid cellular turnover rates, it can be implied that most of the cells within these tissues do not live long enough to accumulate the requisite number of mutations required for malignant transformation.

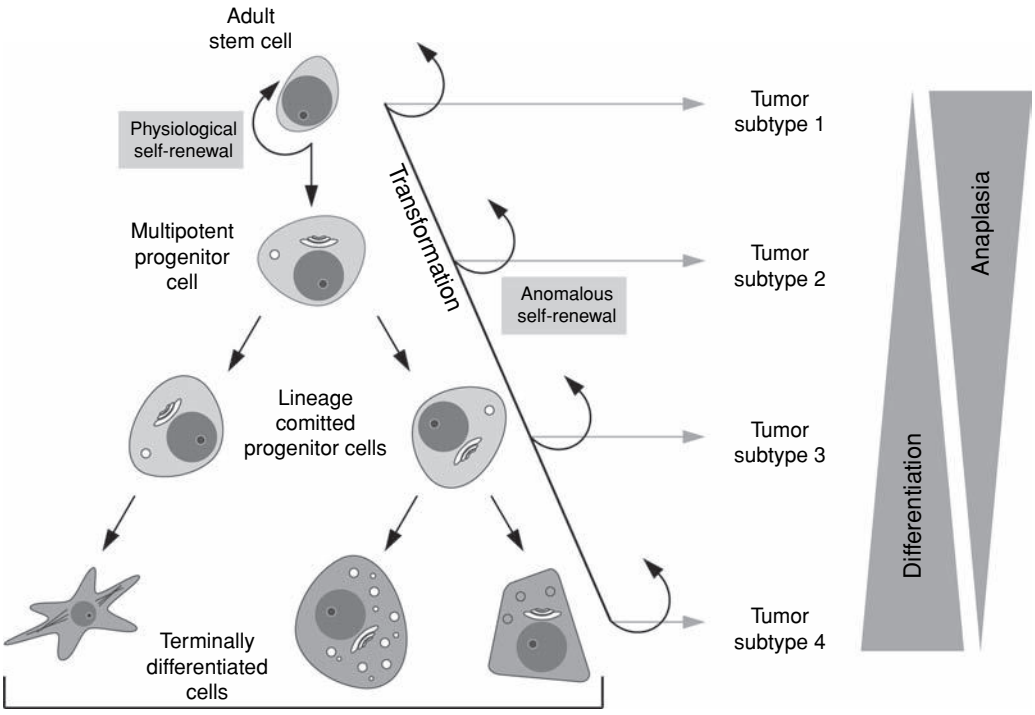


**Figure 4.2** Schematic representation of a normal and a cancer-associated cellular hierarchy.

The long-lived resident tissue stem cell, however, provides an elegant target for mutational transformation. By slowly amassing mutations throughout its lifetime (i.e. the lifetime of the organism), this cell may reach the threshold for tumorigenic conversion. Once transformed, this cell is thought to continue to undergo symmetric and asymmetric division and generate lineage-committed progeny, albeit the hierarchical nature of this differentiation is perturbed (Figure 4.2). The retention of self-renewal capacity only in the CSC is responsible for the limited tumorigenic potential of the cells which comprise the bulk of the tumor. Recent evidence now suggests that lineage-committed progenitors which have gained self-renewal capacity may also be potential sources of CSCs.

## Molecular pathogenesis of cancer

The heterogeneity between and within distinct types of human cancers highlights the diversity in mechanisms responsible for transformation. Indeed, aberrations in hundreds of molecules have been implicated in the causation of various cancers. Despite this variation, most cancer cells exhibit similar aberrations in general cellular processes.

### Anomalous growth factor and growth factor receptor signaling

During development and in physiological contexts, such as wound healing and maintenance of tissue homeostasis, proliferation is regulated by exposure of cells to soluble growth factors within their environment. These factors bind to and activate integral membrane receptors which transduce the mitogenic signal across the plasma membrane and then, through the action of cytoplasmic messengers, into the nucleus. Ultimately, this cascade results in changes in gene expression that promote entry into the cell cycle. Under normal conditions, entry into the cell cycle is tightly regulated by controlling the release of soluble growth factors in a spatially and temporally controlled manner, selectively expressing and localizing growth factor receptors and modulating the activity of intracellular signaling molecules.

Cancer cells commonly induce autonomous mitogenic signaling by hijacking the growth factor signaling machinery. This occurs by: (1) autocrine production of growth factors; and/or (2) enabling activation of the growth factor receptor in the absence of ligand. For instance, in a variety of human epithelial cancers,

overexpression of the epidermal growth factor receptor tyrosine kinases EGFR and ERBB2 enables receptor dimerization and constitutive mitogenic signaling in the absence of ligand [16]. Alternatively, tumor cells commonly secrete soluble growth factors that act in a paracrine manner to induce activation of membrane-bound growth receptors and promote entry into the cell cycle [17].

### Abnormalities in signal transduction molecules

Cells use a number of distinct signaling pathways to control their proliferation, including Ras, Jak-STAT, Wnt, nuclear factor-κB, Notch, Hedgehog and transforming growth factor β (TGFβ) [18–24]. These pathways enable cells to receive extracellular signals that then pass through a series of cytoplasmic signal-transducing proteins to activate transcription factors and subsequent gene expression. In cancer cells, the intrinsic activity, concentration and localization of signaling molecules can be affected. For example, the inactivation of NF1, a GTPase-activating protein, prevents hydrolysis of GTP to GDP by Ras, resulting in a constitutively active Ras signaling pathway and uncontrolled proliferation [25]. Additionally, the inactivation of APC prevents formation of the "destruction complex" containing GSK-3β and its phosphorylation and subsequent ubiquitylation and degradation of β-catenin, which migrates to the nucleus and associates with Tcf/Lef transcription factors to drive proliferation and prevent differentiation [26].

### Cell cycle and checkpoint abnormalities

A cell's decision to divide or to become quiescent is influenced by mitogenic signals in the cell's surroundings. Cells are responsive to extracellular signals from the onset of G1 up to the ***restriction point***. Once a cell has committed to divide, there are several checkpoints within the cell cycle to ensure genomic integrity as well as proper replication of DNA and assembly of the mitotic spindle before the cell can progress through the cell cycle. The pairings of various cyclins and cyclin-dependent kinases (CDKs) enable cells to progress through the cell cycle. Cell cycle regulation depends on cyclin levels and availability during the different cell cycle phases, which fluctuate from one phase to the next. Cyclin D1 levels, however, depend on the input from a variety of mitogenic growth factors. Cyclin-dependent kinase inhibitors also regulate cyclin–CDK complexes and include $p15^{INK4B}$, which increases in response to TGFβ signaling to inhibit cyclin D-CDK4/6 [27], as well as $p21^{Cip1}$ and $p27^{Kip1}$, which increase in response to mitogens acting through Akt/PKB to inhibit cyclin-CDK complexes that form at later stages of the cell cycle [28, 29]. pRb phosphorylation by cyclin–CDK complexes regulate its growth-inhibitory activity. In early/mid G1, when pRb is unphosphorylated or weakly phosphorylated by cyclin D-CDK4/6, it binds E2Fs and prevents transcription. In late G1, pRb becomes hyperphosphorylated by cyclin E–CDK2, which results in its complete functional inactivation [30, 31]. Various mechanisms can deregulate cell cycle progression and converge on pRb, including inactivation of the *RB1* gene by mutation or promoter methylation, by gene amplification of cyclin D1, by point mutations in CDK4, or by loss of CDK inhibitors [32]. Additionally, the Myc oncoprotein, acting with Max, induces expression of cyclin D2 and CDK4 in early/mid G1 and of CUL1 and E2Fs in late G1. By associating with Miz-1, Myc represses expression of CDK inhibitors to overcome the growth-inhibitory effects of TGFβ (Figure 4.3) [33, 34].

### Defective cell death

In rapidly dividing tissues, parenchymal cells are born from asymmetric division of a stem/progenitor cell, differentiate and then undergo programmed cell death. The timely death of these cells ensures normal tissue homeostasis by limiting the accumulation of terminally differentiated cells and eliminating senescent and damaged cells. Defects in ***apoptosis***, a form of programmed cell death, are widely observed in various human cancers. It is now readily apparent that cancer results not only from exaggerated cellular division, but also from a failure of normal cells to undergo physiological elimination. The molecular anomalies that are responsible for defects in cell death are diverse, but generally represent alterations that interfere with the activators and/or effectors of apoptosis molecules or those that provide survival signals in the context of pro-death signaling.

Human follicular lymphoma presents an elegant example by which human cancer cells interrupt normal cellular turnover. In this malignancy, the t(14;18)(q32;q21) chromosomal translocation is widely observed and is the causative genetic lesion that induces overexpression of Bcl-2 [35]. This protein, an anti-apoptotic member of the Bcl-2 family of proteins, inhibits the classical mitochondrial apoptosis pathway

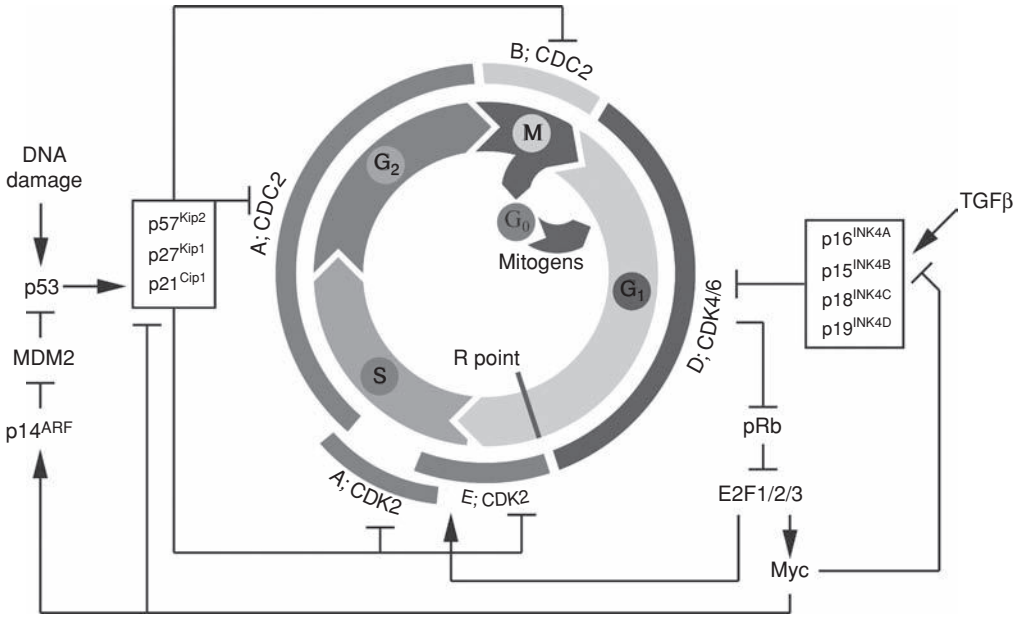


**Figure 4.3** Schematic representation of the cell cycle and checkpoint regulatory system.

[36]. This genomic alteration results in accumulation of B lymphocytes that are unable to proceed through programmed cell death. Additional genetic aberrations that prevent apoptosis and/or promote survival have been described in a number of human cancers [37].

### Aberrant differentiation

Mammalian organogenesis proceeds through an elegant hierarchical establishment of cellular and functional components. Each step of this process entails an increasing degree of cellular commitment that is driven by changes in transcriptional and/or epigenetic programs. The end-product of this process is the formation of terminally differentiated cellular components that are specialized to perform specific biological functions. In normal tissue morphogenesis, this process is tightly regulated by growth factors, morphogens and signals derived from cellular and noncellular components of the microenvironment. The CSC theory of cancer operates under the premise that cells within this hierarchical arrangement are targets of transforming mutations and that these different cellular origins account for the heterogeneity observed in human cancers. Well before the CSC theory gained popularity, dysregulated growth patterns and impaired differentiation, termed ***dysplasia*** and ***anaplasia***, repectively, were widely recognized in human cancers. Emerging evidence suggests that defects in cellular fate commitment and the existence of aberrant cellular hierarchies within human cancers contribute to malignancy.

Acute promyelocytic leukemia (APL) is an early-onset myeloid leukemia whose pathogenesis is entirely related to failed differentiation. The t(15;17)(q22;q12) translocation is observed in over 95% of APL cases and results in a reciprocal translocation of the promyelocytic leukemia gene (*PML*) with the retinoic acid receptor-alpha gene (*RARA*) [38, 39]. Retinoid signaling plays a critical role in differentiation of promyelocytes into mature granulocytes. Aberrations in RARA cause a maturation arrest of promyelocytes and result in the accumulation of these immature cells within the peripheral blood. Administration of all-*trans* retinoic acid (ATRA) causes the terminal maturation of promyelocytes and has transformed this rapidly fatal and incurable malignancy into a disease in which clinical remission is almost universally achieved [40].

### Failure of DNA damage repair systems

Intact DNA damage repair systems are critical to maintain genomic stability and prevent tumorigenesis,

as demonstrated by studies of patients with inherited defects in DNA-repair genes, such as xeroderma pigmentosum (defect in nucleotide excision repair [NER]), Nijmegen breakage syndrome (defect in *NBS* and processing of double-stranded DNA [dsDNA] breaks), ataxia telangiectasia (defect in *ATM* and homology-directed repair [HDR] of dsDNA breaks) and familial breast and ovarian cancers (defect in *BRCA1* or *BRCA2* and HDR of dsDNA breaks). DNA damage can result from endogenous biochemical processes (e.g. depurination, deamination, oxidation) and exogenous carcinogens (e.g. radiation, alkylating agents, heterocyclic amines). Cells employ a variety of mechanisms to repair DNA damage, including mismatch repair (MMR) enzymes that recognize normal but misincorporated nucleotides and other enzymes that detoxify (e.g. glutathione-S-transferase) or remove DNA adducts (e.g. $O^6$-methylguanine DNA methyltransferase). Additionally, base excision repair (BER) recognizes chemically altered bases caused by endogenous sources, NER recognizes helix-distorting adducts from exogenous sources, HDR of dsDNA breaks utilizes the undamaged, homologous DNA sequence to direct repair and the more error-prone non-homologous endjoining (NHEJ) results in fusion of two dsDNA ends [41]. One anti-cancer treatment approach takes advantage of the failure of DNA damage repair. Poly (ADP-ribose) polymerase (PARP) is an enzyme that participates in BER, a mechanism that cancer cells with mutations in *BRCA1* or *BRCA2* depend on to repair their DNA damage. These cancer cells become hypersensitive to chemotherapy and radiation when PARP inhibitors are given to prevent repair by this mechanism as well [42].

## Hallmarks of cancer

Despite the tremendous molecular heterogeneity observed in human cancers, malignant cells exhibit a common set of behaviors regardless of their tissue of origin or the specific genomic insults that they have sustained. These ***hallmarks of cancer*** [43] (Figure 4.4) are the biological manifestations of the molecular anomalies that exist within cancer cells.

### Self-sufficiency in growth signaling

Normal growth factor receptor signaling begins with cognate ligand-receptor binding followed by cytoplasmic transduction of the signal into the nucleus and transcription of genes that stimulate cell proliferation and cell growth. By the mechanisms described in the "Anomalous growth factor and growth factor receptor signaling" section above, cancer cells have evolved mechanisms that deregulate growth signaling and enable them to become self-sufficient [43].

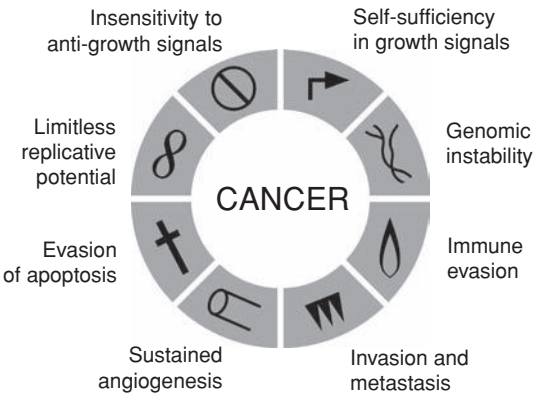


**Figure 4.4** The hallmarks of cancer.

### Insensitivity to anti-growth signals

Just as cells respond to growth-promoting cues within their microenvironment, they also sense and react to molecular signals which antagonize proliferation. When grown in vitro, normal cells proliferate until a confluent monolayer is formed and then become quiescent. This phenomenon of ***contact inhibition*** implies that high cell density and/or extensive contact with neighboring cells restrains further cell growth. Cells that ectopically express certain viral or cellular oncogenes and cells derived from established tumors lack contact inhibition. After these transformed cells reach confluence, they appear to ignore the anti-growth signals that constrain normal cells and begin piling upon one another in a disorganized fashion.

We now understand many of the molecular signaling cues that are ignored or otherwise misinterpreted in malignant cells. In normal epithelial cells, TGFβ family members appear to be largely responsible for conferring anti-growth signals [44]. Exposure of cells to TGFβ results in increased expression of $p15^{INK4B}$ and $p21^{Cip1}$ (Figure 4.3) [27, 45]. In addition, activation of Smad3 by a ligand-bound TGFβR results in formation of a Smad3-E2F4/5-p107 trimer that potently inhibits expression of Myc [46]. Cancer cells almost universally find ways to evade the anti-growth signals mediated by TGFβ. Mutational inactivation of the *RB1* gene is extremely common

in human cancers and largely compromises the anti-growth effects of TGFβ because release of E2F1/2/3 is no longer dependent upon formation of a cyclin D-CDK4/6 complex (Figure 4.3). Alternatively, cancer cells have been demonstrated to mutate the negative regulatory sequences in the *Myc* promoter, resulting in constitutive expression of this gene, and to inactivate the genes encoding the Smad transcription factors or the TGFβ receptors, both of which are necessary to transduce anti-growth signals into the nucleus [43].

### Limitless replicative potential

Mortality is an intrinsic property of normal cells that is mandated by the unique structure of the ends of our linear chromosomes. Molecular limitations in eukaryotic DNA polymerases result in the progressive shortening of chromosomes, which when unopposed, leads to a state of ***senescence***. In this state, cells remain alive and metabolically active, but no longer enter into the cell cycle, even in the presence of growth factors. Serial passaging of cells in vitro revealed that normal cells undergo a finite number of divisions and then stop dividing. Cancer cells, however, have the capacity to proliferate indefinitely in vitro. This fundamental difference implies that the acquisition of ***immortality*** is critical to the success of an incipient cancer.

Landmark discoveries by Barbara McClintock revealed that specialized structures at the ends of linear chromosomes termed ***telomeres*** protect against chromosomal fusion events [47]. A more evolved understanding of DNA replication in eukaryotes also revealed that these repetitive structures prevent loss of genomic coding sequence through DNA replication. Because normal somatic cells largely lack the ability to combat the gradual loss of telomeric sequence caused by DNA replication, they are fundamentally restricted in the number of cell divisions through which they can proceed. Certain normal cell types, such as germ cells, express the enzyme ***telomerase***. This ribonucleoprotein enzyme complex catalyzes the addition of repetitive telomeric sequence to the ends of linear chromosomes, thus increasing cellular replicative potential. The finding that most normal cells lack appreciable expression of telomerase while 85–90% of all human cancers aberrantly express this gene highlights the importance of evading replicative senescence and achieving immortality [48]. The remaining 10–15% of human tumors that do not express telomerase achieve immortalization by lengthening their chromosomes using the repetitive telomeric sequence on other chromosomes as a template in a telomerase-independent pathway termed alternative lengthening of telomeres (ALT) [43, 49].

### Evasion of apoptosis

Inactivation of the apoptotic machinery permits the survival of cells with accumulating mutations and promotes evolution of premalignant to malignant cells. One strategy that is frequently encountered is inactivation of the p53 pathway. This can result from mutations in the *TP53* gene itself (which normally induces pro-apoptotic genes), by deletion or promoter methylation of the *ARF* gene (which normally blocks MDM2 action) or by overexpression of MDM2 (which normally blocks p53 action) (Figure 4.3). Additionally, other components of the apoptotic machinery are altered, including promoter methylation of the *APAF1* gene (which normally assembles with cytochrome c to form the apoptosome and activate caspase 9), inactivation by mutation of the pro-apoptotic *BAX* gene and increased expression of the pro-survival *BCL2* gene. Finally, hyperactivation of the PI3K–AKT/PKB pathway by *PTEN* inactivation (phosphatase which removes the 3' phosphate group from $PIP_3$ that was added by the kinase PI3K), or IGF-1/2 overexpression (which activates PI3K) also allows cancer cells to acquire resistance to apoptosis [43].

### Sustained angiogenesis

A tumor's ability to attract blood vessels is essential for its continued growth, otherwise tumor cells located further from the vasculature experience hypoxia and may enter apoptosis or become necrotic. The *Rip-Tag* transgenic mouse model of islet cell tumor progression has provided researchers a way to study this ***angiogenic switch***, which involves the heterotypic interactions among the premalignant islet cells, inflammatory cells and endothelial cells [50]. The islet cells produce vascular endothelial growth factor (VEGF) that is then sequestered by the surrounding extracellular matrix (ECM) as well as still-unidentified signals that recruit mast cells and macrophages. The inflammatory cells produce ***matrix metalloproteinase*** (MMP) 9 that cleaves specific components of the ECM to release VEGF for signaling. The endothelial cells then proliferate and form the tumor vasculature in response to activated VEGF. However, the tumor-associated endothelial cells tend to form a poorly organized vasculature

with gaps in the walls of the capillaries, leading to leaky vessels that contribute to high hydrostatic pressure within tumors. Normally, the lymphatic vessels would drain the fluid, but any lymphatic vessels that form near the tumor vasculature subsequently collapse from the pressure. This high fluid pressure poses a problem when administering anti-cancer therapy [43, 51].

### Invasion and metastasis

The effects of primary tumors are responsible for only 10% of cancer-related deaths. The most insidious aspect of cancer is its ability to spread through the vascular and/or lymphatic systems and seed distant sites. These ***metastases*** disrupt normal tissue and organ function and are responsible for the remaining 90% of cancer mortalities.

The movement of individual malignant cells from the site of primary disease to a distant site entails a series of complex events. Since the vast majority of life-threatening cancers arise in epithelial tissues, this section will focus specifically on the processes that are involved in the invasion and metastasis of carcinomas, though many of these events are involved in the malignant progression of non-epithelial cancers. At the cellular level, a reprogramming event known as the ***epithelial-mesenchymal transition*** (EMT) is thought to play a fundamental role in allowing epithelial cells to acquire motile behavior. This process is associated with repression of epithelial adhesion markers (especially E-cadherin), reorganization of the cytoskeleton, increased migratory and invasive potential and resistance to anoikis [52]. Interestingly, TGFβ signaling appears to play a critical role in induction of the EMT [53]. Cancer cells appear to thwart the growth-inhibitory effects of TGFβ, while preserving their responsiveness to the pro-tumorigenic activities of TGFβ [54]. Acquisition of a mesenchymal phenotype also results in expression of MMPs which enable localized invasion beyond the basement membrane into the underlying stroma. The stromal environment contains both blood and lymphatic vessels that offer the incipient metastatic cell access to the systemic circulation. The process by which cells enter either blood or lymphatic vessels is commonly termed ***intravasation***. After gaining access to the systemic circulation, cancer cells may very well be eliminated before they reach their final destination. Without the ability to attach to a basement membrane and without exposure to mitogenic and/or trophic factors provided by the stroma, cells may die by anoikis. Furthermore, epithelial cells that enter the blood stream will encounter substantial physical shear forces that may destroy them. Those few cells that resist death and withstand the unwelcoming environment of the systemic circulation will lodge in small capillary beds. Through various mechanisms, including those that enabled the cell to move through the stroma and invade the vasculature, these cells can leave the vascular lumen through a process termed ***extravasation***. The last step of the metastatic cascade, termed ***colonization***, represents the most difficult step in the incipient metastatic cell's journey. A variety of growth and trophic factors dictate the sites in which disseminated cancer cells will ultimately establish new tumors. Cells which leave the vascular system and arrive in a location without appropriate stromal support will likely fail to establish clinically significant metastases. Those cells which arrive at an anatomic location that is amenable to their growth will take hold and establish metastatic growths [55]. While a primary tumor in an organ like the mammary gland presents little intrinsic danger, these tumors commonly seed metastases in the brain, liver and lungs. Additionally, a number of epithelial cancers commonly metastasize to bone and result in significant pain. Clinically detectable metastases very commonly compromise structure and/or function of these vital organs and ultimately cause much of the morbidity and mortality observed in clinical oncology.

### Genomic instability

A cell has a variety of mechanisms to ensure the structural integrity of its DNA, including enzymes that detoxify mutagenic molecules and proteins that recognize and repair the damage. Cancer susceptibility increases when these repair processes are affected. This can occur by inherited or somatic mutation of key proteins or epigenetic alterations such as promoter methylation that leads to functional inactivation of key proteins. For example, MMR defects prevent the detection and repair of sequence mismatches in genes, such as the type II TGFβ receptor that have microsatellite repeats in their sequences. In this case, it introduces a nonsense mutation, which results in a truncated receptor that can no longer function in its growth-inhibitory signaling. Changes in chromosome structure as a result of translocations or fusions of unrelated chromosomes as well as changes in chromosome

number as seen with altered *MAD1* or *MAD2* (both involved in the spindle assembly checkpoint that leads to aneuploidy) also appear to drive tumor progression [56].

## Basic immunology

The immune system has two major features: immune recognition and immune response. Immune recognition is the ability to distinguish foreign invaders from self-components, which then leads to the immune response in which the foreign invader (or non-self component) is eliminated. The immune response to a foreign invader involves both innate immunity and adaptive immunity. Innate immunity is the body's first line of defense against a foreign invader. The ***innate immune response*** is rapid and fixed and includes anatomic barriers (e.g. skin), inflammation and soluble molecules (e.g. interferons [IFNs]). The major cellular components of an innate immune response include neutrophils, macrophages, dendritic cells and natural killer (NK) cells. The ***adaptive immune response*** is the body's second line of defense that develops in response to a foreign invader. The adaptive immune response is a delayed response that demonstrates antigen specificity, diversity, immunological memory and self/non-self recognition. The adaptive immune response can be further divided into ***humoral*** and ***cell-mediated*** responses. In an adaptive humoral immune response, B lymphocytes interact with their specific antigen (pieces of a foreign invader); differentiate into plasma cells, which then secrete antigen-specific antibodies. The secreted antibodies bind to their specific antigen and facilitate clearance of that antigen. In an adaptive cell-mediated immune response, T lymphocytes recognize their specific antigen, resulting in the secretion of various cytokines. Cytokines direct numerous cellular activities, including activation of T helper ($T_H$) or T cytotoxic ($T_C$) lymphocytes, leading to cell-mediated toxicity. The major cellular components of the adaptive immune response are T lymphocytes, B lymphocytes, NK cells and antigen-presenting cells (e.g. dendritic cells).

### Tumor immunology

The immune system's ability to distinguish self- from non-self components and react only to non-self components is crucial to an appropriate immune response. Failure in the ability to distinguish self from non-self can result in autoimmune reactions or facilitate the development of a tumor [57]. The immune system's role in tumor development can be defined in three parts [58]:

1. Protect the body from viral infections thereby reducing any virus-induced tumors.
2. Eliminate foreign invaders and resolve inflammation thereby eliminating an environment that can be conducive to tumorigenesis.
3. Identify and eliminate tumor cells on the basis of tumor-specific antigens or molecules induced by stress.

There is both clinical and epidemiological evidence that suggests a strong association between chronic infection, inflammation and cancer [59]. For example, infection with *Helicobacter pylori* is associated with gastric carcinoma and chronic viral hepatitis is associated with hepatocellular carcinoma [60–62]. In animals, if certain components of the immune system are knocked out, there is an enhanced susceptibility to tumor formation [63]. The tumor microenvironment would also indicate there is a role for the immune system in tumor progression. Tumor-infiltrating lymphocytes (TIL), NK cells and NK T cells are found to be associated with tumors. Early on, an increased number of immune cells associated with a tumor is usually correlated with improved prognosis for numerous different tumor types [64]. Therefore, understanding how the immune system recognizes and eliminates transformed cells is crucial to the development of effective anti-tumor therapies.

In 1891, William Coley was the first to attempt to harness the immune system to treat a patient with cancer [65]. Coley noted that some of his patients with sarcoma had spontaneous regression of their tumors and this correlated with a bacterial infection. Coley then used the bacteria to infect cancer patients and, in some cases, complete tumor regression was achieved. In 1909, Paul Ehrlich proposed the idea that the immune system scans for and eradicates transformed cells before they manifest clinically [66]. This hypothesis was extended in the 1950s by Lewis Thomas and Frank MacFarlane Burnet, who proposed that T lymphocytes are the sentinels in the immune response to cancer and coined the term ***immune surveillance*** [67, 68]. Immune surveillance suggests the immune system is on constant alert against transformed cells. In 2002, Robert Schreiber and Lloyd Old extended the immune surveillance theory to indicate that the immune system

does play a role in cancer development and updated the hypothesis to ***immunoediting*** [69–71]. Immunoediting includes the three E's – elimination, equilibrium and escape (Figure 4.5). Elimination is the phase in which nascent tumor cells are destroyed by elements of the innate and adaptive immune response. Equilibrium is the phase in which tumor cells are able to persist, but are equally destroyed by the immune response. However, in the escape phase, due to a number of mechanisms, the tumor disables the immune response and tips the balance in its favor in order to grow and invade. Tumor immune evasion mechanisms include repression of tumor antigens or major histocompatibility complex (MHC) Class I molecules in order to hide their identity, expression of T-cell inhibitory costimulation molecules, induction of T-regulatory cells (Tregs), repression of NK-cell activation ligands (e.g. NKG2D) and induction of apoptosis in immune cells through the release of soluble Fas ligand (FasL) or inhibitory cytokines like interleukin-10 (IL-10) [72–74].

During the elimination and equilibrium phases, TILs may be responding to tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs). The TSAs are specific to a tumor or type of tumor. These antigens are encoded by genes exclusively expressed by the tumor. For example, mutations in the $p14^{ARF}$ and $p16^{INK4A}$ genes result in an epitope found to activate T cells in melanoma [75]. An immune response to a TSA is typically effective. In contrast, TAAs are either expressed only at certain stages of differentiation or are normal gene products that are overexpressed in particular tumors. For example, the oncofetal protein carcinoembryonic antigen (CEA) is typically only expressed early in embryonic development, but is expressed by 90% of colorectal cancers and contributes to the malignant attributes of a colon cancer [76]. The immune response to TAAs is typically low because these proteins still look like self to the immune system.

However, the immune system can also be a detriment in tumor development. Tregs are understood to inhibit autoimmune reactions, prevent further expansion of activated T cells and impede anti-tumor immunity [77]. In cancer patients, Tregs are present in increased numbers in peripheral blood, malignant effusions and tumor tissues [78]. Tumor cells have the ability to secrete CCL22 and mediate the trafficking of Tregs to the tumor via CCR4 [79]. Recruitment of Tregs results in the inhibition of effector T cell activation and function, immune escape and tumor progression.

## Immunotherapy

Manipulation of the immune system is an attractive approach for treatment of many cancers. There are numerous immune mechanisms to exploit, including passive cellular, passive humoral, active specific and non-specific mechanisms. The goals would be to stimulate anti-tumor response, decrease suppressor mechanisms and/or increase tumor immunogenicity [80].

Adoptive T-cell therapy is an example of passive cellular immunotherapy. Adoptive T-cell therapy involves the infusion of T cells derived from autologous or allogeneic sources with the goal of eliminating a tumor and preventing its recurrence [81]. T cells can be stimulated and expanded *ex vivo* prior to infusion in order to enhance their ability to react to a tumor. T cells can also be genetically engineered to express chemokine receptors to promote trafficking to the tumor [82].

Passive humoral immunotherapy includes administration of exogenous monoclonal antibodies. These antibodies can block function, enhance function or be conjugated with toxins or radioisotopes. For example, antibodies to immune inhibitory signals such as PD-L1 or CTLA4 prevent the repression of the immune response and have been shown to enhance tumor regression in mice [83].

Active specific immunotherapy involves infusion of autologous or allogeneic tumor cells with the goal to enhance anti-tumor response. This mechanism also includes ex vivo loading of dendritic cells with tumor antigen followed by infusion into a cancer patient [84]. Further studies have demonstrated that dendritic cells which have been engineered to express transgenic tumor antigens or chemokines are more potent inducers of anti-tumor immunity when compared to loaded dendritic cells alone [85].

Non-specific immunotherapy includes administration of cytokines such as interferon-γ (IFNγ), IL-2 or IL-12 in order to activate macrophages and NK cells and enhance anti-tumor activity [86]. Synthetic CpG oligodeoxynucleotides (CpG ODNs) are agents that have been demonstrated to stimulate both innate and adaptive immunity by enhancing antibody dependent cellular cytotoxicity (ADCC) or serving as an adjuvant to elicit an anti-tumor immune response [87].

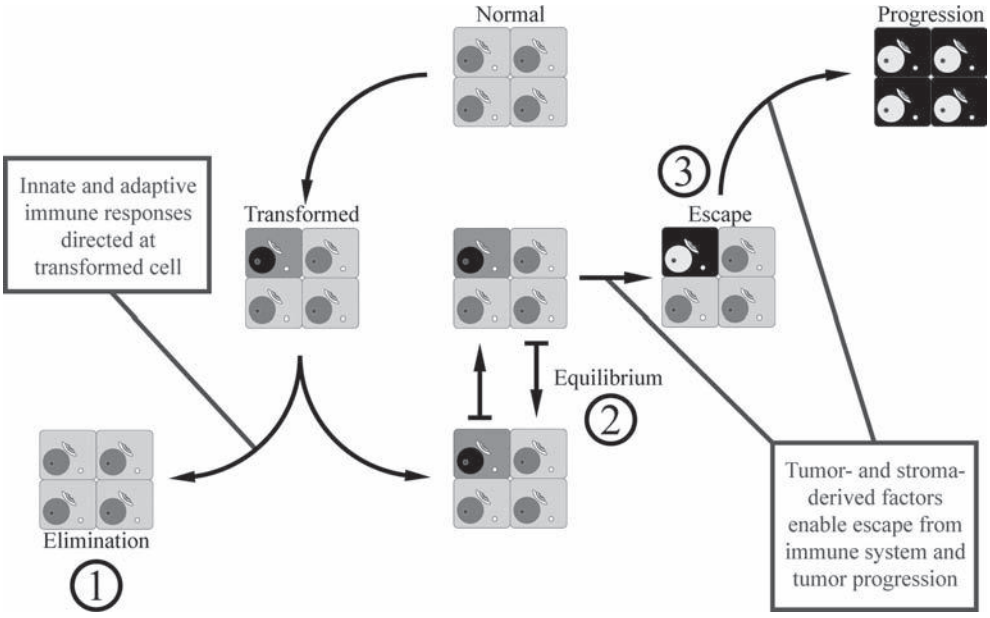


**Figure 4.5** The three E's of immunoediting.

A major frustration in effective immunotherapy regimens are Tregs. However, there are a number of mechanisms that have been demonstrated to decrease Treg activity, clearing the way for an effective anti-tumor immune response. For example, low dose cyclophosphamide treatment selectively depletes Tregs, denileukin diftitox (an IL-2-diptheria toxin fusion protein) reduces the percentage of Tregs in the peripheral blood of cancer patients and daclizumab (a CD25 monoclonal antibody) inhibits Tregs in metastatic breast cancer patients who also received a multipeptide cancer vaccine [88].

Harnessing the immune system to generate an effective, long-lasting anti-tumor response is a major challenge. Continued efforts will make attempts to tip the balance towards the elimination or equilibrium phases of immune surveillance (Figure 4.5).

## Concluding remarks

Human malignancies are the result of acquired genetic changes that deregulate normal cellular proliferation, differentiation and death. Though we have now characterized hundreds of genes and molecular processes that underlie the development and progression of cancer, a comprehensive understanding of tumor biology remains a distant reality. The new paradigm of cancer stem cell theory has revealed that many of the mainstay anti-neoplastic therapies that are currently in use are of little benefit in terms of actually curing cancer. Oncologists and cancer biologists may need to re-evaluate the ultimate goal of cancer treatment, as many forms of human malignancy may in fact be incurable. For these diseases, perhaps a more realistic and reasonable objective is to reduce tumor burden and prevent progression such that cancer becomes a chronic disease. This very real possibility makes advances in cancer patient care and survivorship all the more necessary. Towards this end, major advances in reproductive endocrinology, reproductive biology and anti-neoplastic therapies are needed to ensure that those diagnosed with malignancy before or during their reproductive years have the opportunity to reproduce successfully.

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Chapter 5

# Breast cancer and fertility preservation

## A view from oncology

Carol Fabian and Jennifer Klemp

## Introduction and scope of the problem

It is estimated that 5–7% of cases of invasive breast cancer (~11 000/year) occur in women who are under age 40 at diagnosis [1–3]. The majority of these cases occur between the ages of 30 and 40 [1–3]. As 22% of the first live births in this country occur in women between the ages of 30 and 40 [4], many young women with newly diagnosed breast cancer will not have yet had the opportunity to have a first child. Other women, who may have previously successfully conceived, may not yet have had the opportunity to complete their family. In a survey of women under age 40 at the time of their breast cancer diagnosis, 56% indicated that they wanted to have 1 or more children in the future [5]; however, <10% of women have children after a diagnosis of invasive breast cancer [6–8]. While this low rate of births is likely due to a number of factors, receipt of adjuvant systemic therapy plays an important role for the majority of premenopausal women with breast cancer.

Breast cancer is likely to be self-detected in women under the age of 40 [5, 9], and two thirds of these young women have a stage II or higher tumor [5, 9]. Even for those with early stage disease, the prognosis for women under 40 years with breast cancer is worse than for older women [10, 11]. Achievement of amenorrhea is known to reduce recurrence and improve survival [12–14]. The likelihood of later stage disease, worse prognosis and the combined effects of anti-hormonal and chemotherapy make it likely that most women under 40 years will receive treatment, resulting in depletion of ovarian follicles and a reduced ability to conceive. Treatment-induced preclusion of later childbirth is likely to foster grief and impact later quality of life, particularly if there was little discussion regarding alternatives [15–18]. Women under 40 years of age are likely to be offered genetic counseling and testing, and this is increasingly performed prior to definitive surgery. The emergence of neoadjuvant chemotherapy shortly after biopsy and before definitive surgery for women with stage II or higher tumors increases the complexity and reduces time for standard fertility preservation procedures such as controlled ovarian stimulation (COS) and oocyte retrieval. How can decisions regarding fertility preservation be incorporated into the already crowded and emotionally charged interval immediately following diagnosis?

In this chapter we will cover those issues most likely to be raised by young women who have been recently diagnosed with breast cancer or those at high risk of the disease contemplating assisted fertility procedures.

Questions likely to be posed by women with a recent cancer diagnosis are: (1) Are the benefits from chemotherapy worth the possible loss of fertility? (2) What are the chances that the planned treatment will result in menopause or the loss of ability to become pregnant? (3) If I am still able to conceive will a subsequent pregnancy alter my prognosis? (4) If you alter my treatment to help preserve fertility will it change my prognosis? (5) How much do fertility preservation procedures cost and are they covered by insurance? (6) How is fertility preservation orchestrated with the rest of my treatment? Young women at increased risk of breast cancer want to know if ovarian stimulation is likely to further increase their already elevated risk of breast cancer. All members of the treatment team should be able to provide at least a general response to these questions.

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
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## Risk of recurrence and reduction with adjuvant therapy

Although many young women are interested in fertility preservation, breast cancer cure is generally their number one priority.

The risk of recurrence and death is determined by tumor size, number of involved nodes, biological characteristics of the tumor and length of follow-up. The 15-year risk of recurrence and death for women under age 50 who do not receive adjuvant systemic therapy is 53 and 42%, respectively [19]. Mortality rates at 5 years are about half of that at 15 years. This is primarily because breast cancers (particularly those which are estrogen receptor positive) continue to recur after 5 years from the date of diagnosis and the often prolonged interval between the onset of clinical metastatic disease and death.

The type of adjuvant regimen selected for an individual woman is determined by menopause status, biological characteristics of the tumor and risk of relapse. Biological characteristics with strong impact on treatment selection include estrogen and progesterone receptors (ER and PR), proliferation (usually measured by Ki-67) and presence of the growth factor receptor Her-2 neu. Approximately two thirds of younger women will have hormone receptor positive cancers and one third hormone receptor negative and, in addition, one fifth will have Her-2 neu positive tumors. Approximately one sixth of newly diagnosed women will have a so-called triple negative tumor, which means the cancer does not express ER, PR or Her-2 neu. All women with hormone receptor negative tumors >1 cm in size will be advised to take chemotherapy. Even women with hormone receptor negative tumors <1 cm will often be offered adjuvant chemotherapy if their tumors are high grade and/or Her-2 neu positive. The hormone receptor positive group is very heterogeneous and includes women at one end of the spectrum with very well differentiated tumors which are strongly ER and PR positive with little growth factor receptor expression or proliferation (luminal A). This group is generally offered antihormonal therapy alone in Europe and Canada but in the USA they are also likely to be offered chemotherapy, particularly if they are under the age of 40. At the other end of the hormone receptor positive spectrum are tumors that have a low level of ER or PR and/or have a high proliferative rate and/or express growth factor receptors (luminal B). The latter type of hormone receptor positive tumor has a worse prognosis with anti-hormone therapy alone and chemotherapy in addition is generally advised.

Large meta-analyses of multiple trials with long-term follow-up have been used to assess the effects of systemic therapy on breast cancer outcomes. The most widely referenced is that of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) with the latest major published outcomes in 2005 [19]. Without adjuvant therapy, EBCTCG analyses suggest a 12.5% breast cancer mortality rate at 15 years for women under 50 years with low-risk node negative tumors, 25% for women with high-risk node negative tumors and 50% for node positive tumors. These long-term reported outcomes are reflective of early generation adjuvant regimens such as 5 years of tamoxifen for anti-hormonal therapy and for chemotherapy 6–12 months of cyclophosphamide, methotrexate and fluorouracil (CMF); or 4–6 months of an anthracycline and cyclophosphamide (AC if the anthracycline is doxorubicin); or 6 months of an anthracycline, cyclophosphamide and flurouracil combination (FAC or FEC) (Table 5.1). These early generation regimens with predominately 6 months of treatment reduce 15-year breast cancer recurrence by 39% and 44% in premenopausal estrogen receptor positive (ER+) and estrogen receptor negative (ER–) breast cancer, respectively, and breast cancer mortality by a little over one third. Six months of FAC or FEC is associated with a relative reduction of 44% in 15-year mortality. [19]. Tamoxifen alone reduces mortality by ~30% for ER+ women under age 50 and 40% for those under age 40. For women under age 50 with an ER+ tumor, the addition of 5 years of tamoxifen to an anthracycline-containing regimen was estimated to reduce mortality by a relative factor of 57% [19].

## Amenorrhea and survival

Achievement of amenorrhea appears to be associated with a reduction in relapse and improvement in survival in premenopausal women with ER+ tumors [12, 13, 20]. Amenorrhea need not be permanent to achieve therapeutic benefit. Approximately 2 years of amenorrhea appears to provide the same benefits as permanent amenorrhea [21]. This is an important concept for women and their healthcare providers to understand if they are contemplating fertility preservation. Suppression of ovarian function in women under age 40 with ER+ tumors by adding a gonadotropin-releasing

**Table 5.1** Chemotherapy regimens

| Regimen | Cycles | | Dose | Frequency |
|---|---|---|---|---|
| AC | 4 | Adriamycin | 60 mg/m$^2$ | |
| | | Cyclophosphamide | 600 mg/m$^2$ | Every 3 weeks Dose dense every 2 weeks |
| FAC | 6 | 5 Fluorouracil | 500 mg/m$^2$ | Every 3 weeks |
| | | Adriamycin | 50 mg/m$^2$ | |
| | | Cyclophosphamide | 500 mg/m$^2$ | |
| FEC | 6 | 5 Fluorouracil | 500 mg/m$^2$ | Every 3 weeks |
| | | Epirubicin | 100 mg/m$^2$ | |
| | | Cyclophosphamide | 500 mg/m$^2$ | |
| CEF | 6 | Cyclophosphamide | 75 mg/m$^2$ | D1–D14 |
| | | Epirubicin | 60 mg/m$^2$ | D1 and D8 |
| | | 5 Fluorouracil | 500 mg/m$^2$ | D1 and D8 |
| AC–T | 4 and 4 | Adriamycin | 60 mg/m$^2$ | Every 3 weeks |
| | | Cyclophosphamide | 600 mg/m$^2$ | Dose dense every 2 weeks |
| | | Docetaxel (Taxotere) | 100 mg/m$^2$ | Every 3 weeks |
| | | or Paclitaxel | 175 mg/m$^2$ | Dose dense every 2 weeks |
| TAC | 6 | Docetaxel (Taxotere) | 75 mg/m$^2$ | Every 3 weeks |
| | | Adriamycin | 50 mg/m$^2$ | |
| | | Cyclophosphamide | 500 mg/m$^2$ | |
| CMF | 6 or 12 | Cyclophosphamide | 600 mg | D1 and D8 |
| | | Methotrexate | 40 mg/m$^2$ | D1 and D8 |
| | | 5 Flunoorouracil | 600 mg/m$^2$ | D1 and D8 every 4 weeks |

D, day.

hormone (GnRH) agonist to chemotherapy ± tamoxifen improves disease-free survival by a relative factor of ~25% and may also improve overall survival [19, 22–24]. Use of a GnRH agonist or removal of the ovaries is probably most effective in women who are still menstruating or who have premenopausal levels of estradiol following chemotherapy [25].

## Newer adjuvant treatments and additional benefits

The 2005 EBCTCG analyses probably underestimated benefits from adjuvant chemotherapy and anti-hormonal treatments as long-term data from some of the newer successful systemic therapy regimens are not included. These "newer" regimens include those incorporating: (1) taxanes; (2) intravenous bisphosphonates for women with hormone receptor positive cancer; (3) trastuzamab for women with Her-2 neu positive cancer; and (4) "dose dense" regimens in which the interval between chemotherapy cycles is shortened. Overall, without regard to stage, hormone receptor positivity or menopause status, taxane-containing regimens appear to result in an absolute 3% increase in survival [26]. Taxanes appear to provide incremental benefit when given with anthracyline and cyclophosophamide in women with luminal B, triple negative or Her-2 neu positive cancers. There appears to be no incremental benefit for women with luminal A tumors compared to older regimens [27, 28]. Intravenous bisphosphonates appear to reduce recurrence by about one third in premenopausal women with ER+ tumors given anti-hormonal therapy with tamoxifen and ovarian suppression with a GnRH agonist (i.e. goserelin) [29]. Trastuzamab given with chemotherapy or chemo-hormonal therapy improves relapse free and overall survival by about 50% in women with

Her-2 neu positive tumors compared to chemotherapy or chemo-endocrine therapy alone[30]. Taxane and carbopalatinum regimens appear to be as efficacious as anthracycline cyclphosphamide and taxane combinations when given with trastuzamab to women with Her-2 neu positive tumors [31] and are probably not as likely to result in sterility.

Women often want to know which components of the treatment plan are giving them the greatest benefit in terms of reduction of risk of relapse and death. Understanding the risk/benefit of each component allows for a more critical review of treatment recommendations. Providers generally discuss the likelihood of relapse following local therapy and then estimate the incremental benefit likely to accrue with the addition of each agent. For example, a 35-year-old woman with a 2 cm, node negative, strongly hormone receptor positive tumor, with little proliferation (luminal A) might have a 30% risk of relapse at 10 years. She can expect a 40% relative reduction in risk of recurrence with tamoxifen alone and another 25% relative reduction with the addition of a GnRH agonist, bringing her absolute risk down to 13–14%. An addition of 2 years of an intravenous bisphosphonate could reduce her risk by an additional one third to an absolute value of 9%. Adding polychemotherapy with an alkylating agent might reduce the risk by an additional 20% to an absolute value of 7%. If she does not use a GnRH agonist, however, the incremental benefit of chemotherapy with the subsequent ovarian hormonal suppression would be higher. On the other hand, a woman with a weakly hormone receptor positive tumor or one with a high proliferation rate or presence of growth factor receptors (luminal B) is likely to have less relative reduction of risk of recurrence with anti-hormonal agents and more from cytotoxic chemotherapy. Calculation of the relative risk reduction with an individual intervention is much simpler in women with hormone receptor negative tumors. A woman with a 2 cm, node negative, hormone receptor negative tumor is likely to have a baseline risk of relapse of ~40%. This risk can be reduced by ~40% with chemotherapy if the woman has a triple negative tumor and by ~50% with chemotherapy plus trastuzamab if the woman has a Her-2 neu positive tumor. A number of tools have been developed to facilitate this process including gene expression panels for ER+ women [32–34] and models incorporating age, stage and biomarker characteristics such as Adjuvant Online (http://www.adjuvantonline.com/index.jsp) [35, 36].

## Standard regimens and how they might be altered to help preserve fertility

### Women with ER+ tumors (Figure 5.1)

Premenopausal women with an ER+ or PR+ invasive cancer of any size will receive at minimum 5 years of anti-hormonal treatment with tamoxifen with or without ovarian suppression or ablation, in addition to the addition of 2–3 years of intravenous bisphosphonates. Women with hormone receptor positive tumors with less favorable biological characteristics, such as a high proliferation rate, low expression of ER, low or absent expression of PR or expression of growth factor receptors, are generally offered chemotherapy as well. Women continue to relapse between 5 and 10 years after diagnosis even with 5 years of anti-hormonal therapy ± adjuvant chemotherapy [37]. This late relapse rate for women under 35 years is ~1.5% per year and is higher for women who had positive nodes and/or who are ER+ [37]. Emerging data suggests reduced recurrence when 5 years of an aromatase inhibitor is added to 5 years of tamoxifen for women who were premenopausal at the time of initiation of tamoxifen [38]. Therefore, it has become increasing more common for oncologists to consider giving adjuvant endocrine therapy for a total of 10 years to women with node positive ER+ tumors.

Good prognosis ER+ premenopausal women may not need chemotherapy or may take only four cycles of a cyclophosphamide-containing regimen. Doxorubicin (adriamycin) combined with a taxane and omitting cyclophosphamide appears equivalent to an anthracycline plus cyclophophamide but is probably less efficacious than all three drugs and should be reserved for women with fairly good prognosis [26].

The GnRH agonists or analogues are often used concomitantly with chemotherapy and/or anti-hormonal therapy in young premenopausal women with ER+ tumors to improve relapse free survival [22, 39, 40]. These agents stimulate release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary and in the first few weeks may increase ovarian hormone output [41]. With prolonged use ovarian hormone suppression ensues.

Preclinical studies [42–44] and small observational as well as small randomized trials [45, 46]

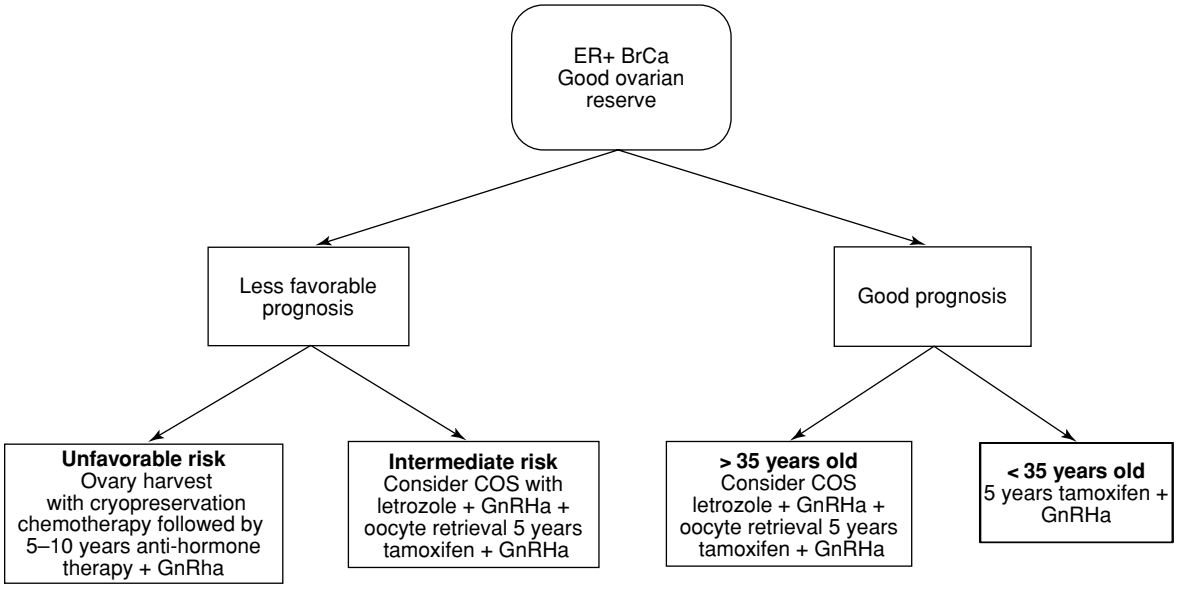



**Figure 5.1** Fertility preservation in women with estrogen receptor positive (ER+) tumors. BrCa, breast cancer; COS, controlled ovarian stimulation; GnRHa, gonadotropin-releasing hormone agonist.

indicate GnRH agonist (goserelin) when given during chemotherapy may also help preserve ovarian function. The recently reported Zoladex (goserelin) in premenopausal patients (ZIPP) study randomized premenopausal women taking six cycles of CMF or no CMF (women with no positive nodes and a small tumor could be randomized to no CMF as well) to concomitant anti-hormonal therapy with tamoxifen alone, goserelin alone, goserelin + tamoxifen or no hormonal therapy. The mean age at diagnosis was 45 years. At 1 year after completed endocrine therapy (36 months from randomization) the proportion of women with amenorrhea was 90% for controls, 87% for tamoxifen, 93% for goserelin + tamoxifen and 64% for goserelin alone ($P = 0.006$) [46]. The mechanisms of action of GnRH/luteinizing hormone-releasing hormone (LHRH) agonists in preserving ovarian function are not understood, but may involve reduced FSH, reduced ovarian perfusion and activation of GnRH receptors with upregulation of intragonadal anti-apoptotic molecules [47]. Many reproductive specialists remain unconvinced that GnRH agonists improve the ability to conceive, although it is possible that they reduce permanent amenorrhea [48, 49]. A large intergroup randomized trial of a GnRH agonist versus placebo in ER– women undergoing chemotherapy is ongoing and will hopefully answer this question. In the meantime for women with ER+ cancers desiring fertility preservation, particularly those who do not undergo oocyte retrieval, use of the GnRH agonist seems reasonable as it is likely to have a therapeutic effect on the tumor even though there may be no protective effect for fertility.

## Women with ER– tumors (Figure 5.2)

Premenopausal women with an ER and PR negative high-grade tumor of 1 cm or more in size, or those with positive nodes, will likely undergo 6–8 cycles of adjuvant chemotherapy in addition to definitive surgery ± local radiation. The chance of permanent amenorrhea is ~40%. Although there is little concern about increasing tumor proliferation with drugs for ovarian stimulation and oocyte/egg retrieval, these tumors are generally rapidly growing, fueling concerns about treatment delay for fertility preservation, especially for women requiring neoadjuvant chemotherapy. The combination of a taxane and carboplatinum or cisplatinum with trastumab appears to be as effective as cyclophosphamide and an anthracycline for most women, resulting in less ovarian toxicity [31, 50]. For women desiring fertility preservation, six cycles of carboplatin and a taxane with trastuzamab can be substituted for chemotherapy with an anthracycline and cyclophosphamide and taxane. Excellent results are also being observed with cisplatin as a single agent

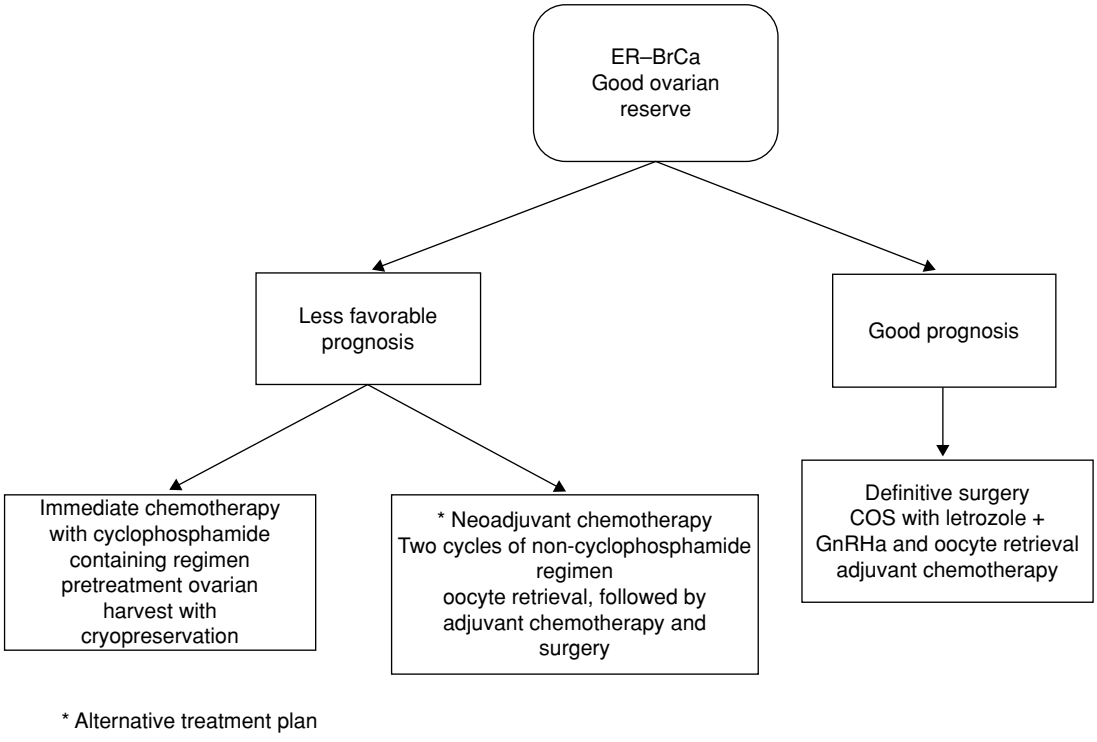



**Figure 5.2** Fertility preservation in women with estrogen receptor negative (ER–) tumors. BrCa, breast cancer; COS, controlled ovarian stimulation; GnRHa, gonadotropin-releasing hormone agonist. *Alternative treatment plan.

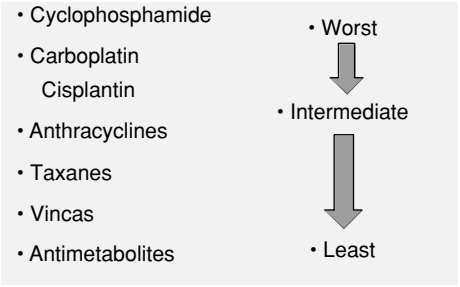
in neoadjuvant trials of women with triple negative tumors [51]. Data are very preliminary at this point and use of cisplatin alone in a woman with a triple negative tumor should only be used in the neoadjuvant "trial" setting with follow-up adjuvant chemotherapy with a standard adjuvant regimen if complete pathological response is not obtained.

In summary, regimens which omit or deliver four or fewer cycles of cyclophosphamide have the greatest chance for fertility preservation. For women planning to take six cycles of cyclophosphamide, flurouracil and an anthracycline (FEC or FAC) or four cycles of cyclophosphamide and an anthracycline followed by four cycles of taxane, pre-chemotherapy oocyte retrieval ± in vitro fertilization may enhance later chance of pregnancy. When this is not possible, ovarian harvest and cryopreservation may be considered. For women with ER+ tumors, use of a GnRH agonist beginning 2 weeks before chemotherapy and continuing during and post-chemotherapy, is advised to reduce the chance of recurrence and enhance prospects of later successful pregnancy.

## Chances that treatment will induce amenorrhea or loss of fertility

The median age at menopause in the USA is 51 years, but the ability to become pregnant is markedly reduced beginning about 10 years before cessation of menses due to depletion of primordial follicles. It is not clear how many premenopausal women wish to become pregnant after a diagnosis of breast cancer, but it is clear that a woman is unlikely to become pregnant if she stops menstruating with treatment and then fails to resume menses. The main determinant of chemotherapy-induced amenorrhea is the age of the woman at the time of diagnosis and the number of cycles of alkylating agent chemotherapy she has received [52, 53]. The alkylating agent cyclophosphamide is one of the oldest and most effective drugs in breast cancer. In general, each cycle of cyclophosphamide chemotherapy is associated with an increase in ovarian age of about 1.5–3.0 years depending on dose and frequency [52–55]. A woman who takes the equivalent of 2.4–3.0 g/m$^2$ of cyclophosphamide

**Table 5.2** Agents associated with amenorrhea




over 12–16 weeks can count on adding an approximate 10 years to her ovarian reproductive age, such that if she is aged 30 at the time her chemotherapy is initiated, her ovarian reproductive age will be the equivalent of a 40-year-old woman when her treatment is completed. While she is likely to resume menses, her capacity for a birth without assisted reproductive techniques will be marginal. The mechanism of ovarian toxicity resulting from chemotherapy is not completely understood, but it is likely to result from apoptotic changes in pre-granulosa cells that subsequently develop into follicles [56, 57]. Recent investigations suggest that women with greater levels of neutropenia (often associated with higher doses and more cycles) are more likely to have permanent amenorrhea as are women with certain single nucleotide polymorphisms in enzymes responsible for cyclophosphamide metabolism [58, 59]. Drugs more and less likely to be associated with amenorrhea are given in Table 5.2.

Rates of amenorrhea that have been observed with regimens given to good-prognosis node negative premenopausal women are ~33% for 6 cycles of CMF and 4 cycles of AC either dose dense or non-dose dense (there are no published rates for the 4 cycle taxotere and cyclophosphamide combination) [60–65].

Rates of amenorrhea for chemotherapy regimens often given to high-risk node negative or node positive women such as 6 cycles of FEC or FAC, 6 cycles of AC or 4 cycles of AC followed by 4 cycles of taxotere are generally double that of the good prognosis regimens ranging from 50–65% [66–70]. However, it appears that giving only three cycles of FEC followed by thre cycles of taxotere may have less ovarian toxicity than six cycles of FEC [20]. Fifteen to fifty percent of women younger than age 40 at diagnosis will eventually resume menses. Recovery rates are higher for regimens with less total cyclophosphamide. Amenorrhea is likely to be permanent in 90% of women aged over 40 and in 95% of women aged over 45 [68, 71]. A number of studies indicate a higher rate of prolonged amenorrhea with tamoxifen use after chemotherapy [14, 68]. There is little available data on fertility with platinum alone or a taxane and carboplatin. However, approximately 50% of women receiving adjuvant platinum and fertility-sparing surgery for ovarian cancer were able to successfully bear children [72].

## Pregnancy following a breast cancer diagnosis and risk of relapse

Becoming pregnant after a diagnosis of breast cancer does not appear to result in worse outcomes in case control studies or cohort studies [3, 5, 6, 74]. In fact, in several series pregnancy after a diagnosis of breast cancer appeared to result in a reduced risk of relapse [73–75], particularly for women who waited for 2 years after diagnosis to conceive [75]. At least for some series, this may be due to better prognostic features in women who subsequently had a pregnancy [5]. In only 1 series was a 77% reduction in risk of death observed [74]. For women at high risk of relapse at diagnosis, most relapses appeared to occur within 5 years of diagnosis [74].

## Barriers to fertility preservation for premenopausal women with breast cancer

There are three main barriers to implementing fertility preservation in women with breast cancer: cost; concern about treatment delays; and concern that increasing sex hormones as a result of COS protocols will stimulate proliferation in ER+ tumors. The recent discovery of LHRH receptors even in triple negative tumors [76] and the paracrine interactions between growth factors and estrogen have made many clinicians reluctant to sanction COS in women with recently diagnosed ER– breast cancer, especially if they are to undergo neoadjuvant treatment and the tumor is still in place.

## Cost of fertility preservation procedures

The cost of fertility preservation procedures is covered elsewhere in this volume and will vary by institution, but at our institution the cost of oocyte retrieval, fertilization and cryopreservation is ~$7000 (medication

cost not included) and ovarian harvest and cryopreservation is $6000. Other procedures are available but are rarely utilized at present [77]. Costs for fertility preservation are not covered by most insurance carriers and so it may be difficult for young women to come up with funds on short notice. In addition, in order for embryo cryopreservation to occur, there must be both egg and sperm available and for many single women this adds an additional short-term challenge.

## Fertility preservation and adjuvant therapy delay

For women undergoing surgery first, fertility preservation should not cause significant delays as long as the surgeon sends the woman for a fertility preservation consultation at the time she is initially seen. This is most likely to occur in a multidisciplinary treatment environment where all members of the team have been educated about fertility preservation and a Breast Cancer Survivorship Program or similar facility is readily available to the woman and family to expedite necessary consultations. Controlled ovarian stimulation and oocyte retrieval requires about a month for the typical long-form regimen utilizing gonadotrophins (FSH or human menopausal gonadotropin [HMG]) for ovulation induction; GnRH agonists to prevent a premature LH surge and oocyte damage; and human chorionic gonadotropin (hCG) for ovulation induction oocyte maturation prior to oocyte retrieval. The GnRH agonists along with gonadotropins may result in estradiol levels that are 10–20-fold higher than are observed in the non-stimulated cycle. For the long form of COS, the GnRH agonists typically begins at least 14 days before the anticipated start of gonadotropins which in turn run 10 days in conjunction with the LHRH agonist. A shorter form of COS which is less likely to result in high levels of estrogen is the use of the GnRH antagonist Cetrorelix on the 7th day of gonadotropin administration followed by recombinant LH or an LHRH agonist on day 10 to induce ovulation. Other regimens including one popularized by Oktay *et al*. uses letrozole beginning on day 2, FSH days 4–10 and the GnRH agonist, leuprolide acetate, days 8–10 with oocyte retrieval day 13 [78].

Even with a well-organized team, women undergoing fertility preservation begin their adjuvant chemotherapy about 12 days earlier than those who are not undergoing fertility preservation proceedures [79].

For the woman with a large ER– tumor who needs to start neoadjuvant treatment before surgery, COS and egg retrieval can be attempted between the second and third cycle of chemotherapy, particularly if a regimen is not employing cyclophosphamide. There is little experience with this approach, however.

## COS, hormone levels and risk of recurrence

The newer regimens using letrozole and a GnRH agonist to prevent the premature LH surge and an LHRH (GnRH) agonist to induce final oocyte maturation do not appear to result in higher hormone levels than a natural menstrual cycle in premenopausal women [79]. A recent series by Oktay's group using this regimen shows no difference in recurrence for premenopausal women undergoing fertility preservation with a median follow-up of 2.5 years, and over two thirds of women in both the control and fertility preservation groups had ER+ breast cancers [79].

## Options other than COS and oocyte retrieval

Ovarian harvest and cryopreservation may be the only reasonable option if pregnancy is strongly desired, COS is not possible prior to full course chemotherapy and donor eggs are not an option. Healthy babies have been borne from orthotopic or heterotopic ovarian transplantation after chemotherapy [80]. Although breast cancer can metastasize to the ovary, to date there is little evidence that tumor cells will be re-implanted in women with early stage disease [81].

## COS and high-risk women

There is an ongoing debate as to whether a possible short-term increase in hormones as a result of assisted ovulation results in an increase in the risk of breast cancer. This question is difficult to answer as many women undergoing fertility assistance may already be at increased risk relative to the population because of null-parity or late age at first live birth. In a recent large cohort study, a 13% higher risk of breast cancer was noted in women with ovulation disorders but there was no significant difference in women undergoing

COS versus those who had not [82]. We are currently conducting a pilot trial of premenopausal women receiving letrozole to induce ovulation to determine whether there is a long-term change in breast tissue proliferation and/or expression of other key genes in women who undergo fertility assistance procedures but do not become pregnant. Although there is no current evidence that assisted ovulation significantly increases breast cancer risk, further research is necessary.

## Logistics and summary

Treatment of young women with breast cancer is increasingly complex and requires a number of consultations and multiple decisions shortly after diagnosis. Adjuvant chemotherapy will be recommended for the majority of these women. Chemotherapy ± anti-hormonal therapy will significantly reduce the chances of later successful childbearing and commonly used regimens are likely to add 10 years to a woman's reproductive age. Most young women will receive adjuvant chemotherapy and can increase their chances of conception by undergoing egg retrieval, fertilization and cryopreservation prior to chemotherapy. Historically, this has been performed in the 4–6 week interval after definitive surgery prior to initiation of systemic therapy Women with a new diagnosis of breast cancer under age 40 are likely to have larger tumors and, currently, are more likely to have a recommendation for neoadjuvant chemotherapy prior to definitive surgery than their older counterparts. They are also more likely to have genetic testing and want results prior to a decision about breast conservation versus mastectomy, which increases the likelihood of use of chemotherapy prior to definitive surgery. Use of neoadjuvant therapy decreases the likelihood of egg retrieval prior to chemotherapy. A variety of methods are being evaluated to increase the likelihood of pregnancy after chemotherapy other than pre-chemotherapy egg harvest and in vitro fertilization including pre-chemotherapy ovarian harvest and cryopreservation; use of an LHRH agonist during chemotherapy; and use of chemotherapy regimens that do not contain the alkylating agent cyclophosphamide. Decisions about optimal therapy requires input from the entire treatment team, but the earlier in the process the woman receives fertility counseling the greater the number of fertility options that are likely to be available without compromise in prognosis. If all members of the treatment team are well-informed and organized to provide an immediate consultation for fertility preservation, the patient will begin to talk with the fertility specialist immediately after diagnosis while she is undergoing staging and meeting with medical, surgical and radiation oncologists, improving the chance of COS without inducing treatment delays. We have found that immediate access to a fertility specialist via an intermediary in our Breast Cancer Survivorship Center in the University of Kansas works well. Regional and national education programs are ongoing, but Breast Diagnostic Centers should strive to have logistical information on how women can access information and/or fertility preservation programs available in their waiting rooms. National organizations devoted to this process include Fertile Hope (http://www.fertilehope.org) and the International Society for Fertility Preservation (http://www.isfp-fertility.org).

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Chapter 6

# Breast cancer therapy and reproduction

Larissa A. Korde and Julie R. Gralow

Breast cancer is the most common malignancy in women in developed countries, excluding cancers of the skin. The American Cancer Society projects that in 2010, 207 090 cases of invasive breast cancer and 54 010 cases of non-invasive breast cancer will be diagnosed in the USA [1]. The National Cancer Institute estimates that 2.5 million women with a history of breast cancer are alive in the USA. While the mean age at diagnosis of breast cancer in the USA is 61 years of age, approximately 10% of women with breast cancer are diagnosed at <45 years of age [1, 2]. Younger women with breast cancer have unique concerns and management issues, including the effects of treatment on fertility and the safety and feasibility of pregnancy following diagnosis and treatment of breast cancer. In addition, the likelihood of an inherited predisposition to breast cancer increases with younger age at diagnosis. Women with *BRCA1* and *BRCA2* mutations have greatly increased lifetime risks of early onset breast cancer, second primary breast cancers and ovarian cancer, and therefore also present a number of complex management issues related to conception and childbearing.

## Breast cancer in young women

Breast cancer risk increases with age, with the highest incidence occurring after the sixth decade of life (Table 6.1 [2]). Incidence is slightly higher among white women in older age groups, but breast cancer in women under the age of 45 is more prevalent among black women. Younger women with breast cancer are more likely to have poor prognostic features, such as larger tumor size, regional lymph node positivity, high nuclear grade, estrogen receptor negativity and inflammatory disease [3, 4]. A recent registry-based cohort study in Sweden found that 5-year survival was poorest among women aged <35 years, despite more aggressive treatment in younger women, and improved with increasing age. The authors concluded that these differences in survival could be attributed to both later stage at diagnosis and to a more aggressive intrinsic biology in tumors diagnosed in younger women.

**Table 6.1** Breast cancer incidence rates per 100 000, age-adjusted to the 2000 US-standard population; 17 Surveillance Epidemiology and End Results (SEER) Cancer Registries, 2002–6

| Age at diagnosis (years) | All races | White | Black |
|---|---|---|---|
| 15–19 | 0.2 | 0.2 | – |
| 20–24 | 1.4 | 1.3 | 1.9 |
| 25–29 | 8.1 | 7.8 | 11.2 |
| 30–34 | 25.6 | 25.3 | 30.4 |
| 35–39 | 58.5 | 58.0 | 64.4 |
| 40–44 | 118.4 | 119.3 | 119.9 |
| 45–49 | 185.3 | 188.1 | 178.7 |
| 50–54 | 229.0 | 234.7 | 224.1 |
| 55–59 | 288.5 | 296.1 | 282.7 |
| 60–64 | 351.9 | 366.8 | 321.4 |
| 65–69 | 394.4 | 416.5 | 351.2 |
| 70–74 | 415.4 | 435.9 | 382.3 |
| 75–79 | 441.9 | 465.7 | 387.6 |
| 80–84 | 428.7 | 447.9 | 376.7 |
| 85+ | 342.1 | 350.5 | 335.8 |

From Horner *et al.* [2], based on November 2008 SEER data submission, posted to the SEER website, 2009.

Women at genetic risk of breast cancer have an increased incidence of early onset breast cancer and a markedly higher cumulative lifetime risk of disease. It is estimated that 5–10% of breast cancers occur in

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women with an inherited susceptibility to cancer [5]. The majority of these are women with hereditary breast ovarian cancer syndrome (HBOC), which is explained by deleterious mutations in the *BRCA1* and *BRCA2* genes, although a number of less common genetic disorders, such as Li–Fraumeni syndrome, Cowden's syndrome and Peutz–Jeghers also include a predisposition to breast cancer [6]. At least half of the diagnoses of breast cancer in women with inherited *BRCA1/2* mutations occur under the age of 50 [7]. Women with *BRCA1* mutations have a 40–90% lifetime risk of breast cancer and a 10–40% lifetime risk of ovarian cancer. Women with *BRCA2* mutations have an estimated 40–50% lifetime risk of breast cancer and a 10–20% lifetime risk of ovarian cancer. In addition, women with *BRCA* mutations have a 40–60% lifetime risk of a second breast cancer [6].

## Pregnancy-associated breast cancer

Women with an early age at first birth and multiparity have a decreased risk of breast cancer compared with nulliparous women and those with late parity. Women with a full-term pregnancy before age 20 have a 50% reduced risk of developing breast cancer compared to those with a first pregnancy after age 35. However, studies of breast cancer incidence in young women demonstrate a transient increase in breast cancer risk in the years following pregnancy [8]. Data suggest that the transient increase in risk peaks at 3–5 years after first delivery and levels off 15 years after delivery. Additionally, the increase in risk appears to be varied in both length and magnitude based on the number of pregnancies (i.e. the magnitude of increase in risk is less in biparous than in uniparous women) [8, 9]. There may also be a synergistic effect between pregnancy and family history of breast cancer. One study suggested that in women with no family history, the transient increase in risk was mainly seen in women with a late age at first live birth, while women with a family history also experienced an adverse effect of pregnancy at younger ages [10]. The mechanism behind these effects is not well understood, though several hypotheses have been investigated. Possible explanations include an effect of increased pregnancy-related hormones such as estrogen, progesterone and growth hormone on previously initiated cells, the immunosuppressive effect of pregnancy or a pro-tumerogenic effect of postpartum and post-lactation breast tissue involution [11].

Concurrent breast cancer is estimated to occur about 1 in 1500 to 1 in 4000 pregnancies [12]. Since more women in the USA are delaying childbearing into their 30s and early 40s, it is likely that this number will increase. The diagnosis of breast cancer during pregnancy is usually prompted by palpation of a breast mass. Appropriately shielded mammography and ultrasound are safe during pregnancy. Breast MRI has not been evaluated due to concern for fetal risk of gadolinium toxicity and heating/cavitation effects [13]. Breast biopsy is indicated for definitive diagnosis.

A recent international collaborative study investigated cancer diagnoses, treatment and obstetric and neonatal outcomes of women with invasive cancer diagnosed during pregnancy [14]. The study included 216 pregnant women diagnosed with cancer between 1998 and 2008; 46% of study participants were breast cancer patients. The mean age at diagnosis of all patients was 33 years. Twenty-four percent of patients were in their first trimester of pregnancy and 43% and 33% were in the second and third trimesters, respectively. More than half of the patients included in this study (55%) received chemotherapy, either alone or in combination with surgery. Among patients receiving chemotherapy during pregnancy, there was a significant increase in preterm labor. In addition, compared with patients that received no treatment, treated patients had a significantly higher risk of delivering a small-for-gestational-age child. This was primarily seen among women treated for hematological malignancy and not seen in women with breast cancer, suggesting that the specific chemotherapeutic agents used for treatment of certain tumors might have a more substantial impact on fetal growth. There did not appear to be an increased risk for physical malformations at birth among children of patients receiving chemotherapy.

As in the non-pregnant state, optimal treatment of breast cancer during pregnancy generally involves both local and systemic treatment. Modified radical mastectomy is the surgical treatment of choice. Sentinel lymph node biopsy during pregnancy has not been well studied. The dose of radiation delivered to the fetus with technetium is estimated to be low, but the use of blue dye mapping during pregnancy is not recommended. Thus, if only one mapping technique is used, the sensitivity of sentinel lymph node mapping may be decreased [15]. Breast-conserving therapy should only be considered in the third trimester as

radiation therapy is contraindicated during pregnancy, and thus can only be safely administered postpartum. Radiation risks to the fetus include a high rate of spontaneous abortion in the first few weeks after conception, an increased risk of congenital malformations between the 2nd and 8th weeks of gestation and an increased risk of mental retardation after the 8th week of gestation. In addition, children exposed to radiation in utero have an increased risk of childhood cancer [16].

Few drugs have been studied in the setting of pregnancy, and thus there are limited safety data on chemotherapeutic agents in pregnant women. Cytotoxic chemotherapy should be avoided during the first trimester of pregnancy due to a high risk of teratogenicity during organogenesis [17]. Two small studies have reported on the use of combination chemotherapy for the treatment of breast cancer during the second and third trimesters of pregnancy. Berry *et al.* reported no birth defects or serious peripartum complications among women treated with 5-flourouracil, doxorubicin and cyclophosphamide at standard doses [18]. Ring *et al.* reported on a series of 27 women treated during pregnancy with either cyclophosphamide, methotrexate and flourouracil or an anthracycline-based regimen at five London hospitals; no birth defects were seen in this study, but one child had intrauterine growth restriction and two had respiratory problems requiring short-term stays in the Neonatal Intensive Care Unit [19]. Taxanes have been even less well studied in pregnancy, with only case reports suggesting safety of the use of docetaxel and paclitaxel during the second and third trimesters [20–22]. The delivery of cytotoxic therapy within 3 weeks prior to delivery is associated with both maternal and infant leucopenia. As a result, chemotherapy should be held beyond 35 weeks of gestation to minimize risk of maternal and infant infection and hemorrhage [15]. Anti-emetics (including 5HT3 serotonin antagonists) are generally considered safe during pregnancy and should be used as necessary [15]. Granulocyte-colony stimulating factors have been used in pregnancy in limited settings [23, 24], and are considered category C during pregnancy (should be given only if the potential benefit justifies the potential risk to the fetus). Trastuzumab use in pregnancy has been associated with oligohydramnios and anhydramnios [25–27]; thus, delay of trastuzumab until after delivery, when feasible, should be considered. Tamoxifen use has been associated with neonatal malformations of the genital tract and craniofacial defects [15]. Although there are reports of patients receiving tamoxifen during pregnancy without damage to child [28, 29], its use in the adjuvant setting should be delayed until after delivery. Teratogenic effects of aromatase inhibitors have been described in animal models [30], but there are no data on their use in pregnancy in humans. The use of gonadotropin-releasing hormone (GnRH) agonists is not advised during pregnancy, although their use in a series of five patients was not associated with teratogenicity [31].

## Effects of breast cancer treatment on reproductive function

While the diagnosis of breast cancer during pregnancy itself is rare, about 10–15% of breast cancers are diagnosed in reproductive-aged women, and up to 3% of breast cancers occur in women of peak reproductive age (25–35 years) [32], many of whom desire maintenance of fertility and post-treatment conception. Young age at diagnosis appears to be an adverse prognostic factor [4], and thus young women are likely to undergo adjuvant systemic therapy, with attendant consequences on fertility. Treatment for breast cancer can impact fertility for a variety of reasons, including a toxic effect of chemotherapy on ovarian follicles, advice to delay pregnancy due to concern for recurrence of disease and the recommendation for 5 years of adjuvant endocrine therapy for hormone-responsive disease, after which age-related decline in fertility is more likely to be an issue. In addition, ovarian ablation or bilateral oophorectomy may be advised for women with hormone-responsive cancer or those with a *BRCA1/2* mutation. This is considered particularly important for the latter group, who also have a significant lifetime risk of ovarian cancer.

### Ovarian function following chemotherapy for breast cancer

The effect of chemotherapy on ovarian function is related to patient age, and to the specific agent and dose used [33]. It is important to note a number of methodological issues arise when assessing fertility potential following breast cancer. While many studies report rates of amenorrhea following chemotherapy, the time point at which menstrual function is assessed various widely among studies. Furthermore, the presence or absence of menses is an imperfect surrogate for

**Table 6.2** Reported rates of amenorrhea with common breast cancer regimens

| Regimen | No. of cycles | Younger[a] women with amenorrhea (%) | Older[a] women with amenorrhea (%) |
|---|---|---|---|
| CMF (cyclophosphamide, methotrexate, fluorouracil) | 3–12 | 18–65 | 74–97 |
| Anthracycline plus alkylating agent (most commonly doxorubicin and cyclophosphamide) | Variable | 32–46 | 73–100 |
| Anthracycline followed by taxane (doxorubicin or epirubicin + cyclophosphamide followed by paclitaxel or docetaxel) | 4 AC/EC → 4 taxane | 6–46 | 35–86 |

Adapted from Walshe *et al.* [36].
[a] In most studies, 40 years of age was used as the cut-point to differentiate younger from older premenopausal women, although women who were 40 years at diagnosis were defined as "younger" in some studies and "older" in others.

fertility, as some women may maintain menstrual function but still have impaired fertility. Pregnancy after cancer treatment is necessarily affected by social factors and patient preference, and thus is an impractical outcome measure and seldom reported; thus, the data described here will focus on amenorrhea. Amenorrhea following chemotherapy may be temporary or permanent, and results from interference of follicular maturation, with or without depletion of primordial follicles [34]. For those who do resume menstruation, data suggest a continued impairment of fertility and an earlier mean age at menopause [35]. A summary of studies presenting effects of specific chemotherapy regimens on menstrual function is shown in Table 6.2 [36].

Chemotherapy regimens that include an alkylating agent (predominantly cyclophosphamide in the treatment of breast cancer) induce high rates of amenorrhea, ranging from 61 to 97% in women over 40 years and from 18 to 61% in women under 40 years [33]. Amenorrhea occurs sooner in older women and is more likely to be irreversible. Higher doses of cyclophosphamide appear to have more of an effect on menstrual function; in one study comparing 12 cycles of single-agent cyclophosphamide (130 mg/m$^2$) to 12 cycles of oral cyclophosphamide, methotrexate, fluorouracil (CMF; cyclophosphamide dose 80 mg/m$^2$) in premenopausal women, the rates of amenorrhea were 70 and 63%, respectively [36].

Anthracyclines have been increasingly incorporated into adjuvant treatment for breast cancer, as studies suggest that 3–6 months of anthracycline-based therapy is equivalent or superior to CMF [37]. While many studies suggest that rates of amenorrhea are lower with anthracycline-based chemotherapy than with CMF, direct comparisons are difficult due to differences among trials in individual agent dosing and the number of cycles given. In one trial comparing six cycles of CMF to six cycles of CEF (cyclophosphamide, epirubicin and fluorouracil), a higher rate of amenorrhea was seen in CEF-treated patients [38]. Conversely, other studies suggest a lesser impact on ovarian function with anthracyclines [39–41]. These differences may be partially attributable to a higher cumulative dose of cyclophosphamide used in the CMF regimen. Patient age is consistently related to both risk and duration of amenorrhea due to anthracyclines. In a joint analysis of three prospective trials using doxorubicin at M. D. Anderson, rates of amenorrhea among women aged 40–49, 30–39 and <30 years were 96, 33 and 0%, respectively. A majority of those >40 years of age at diagnosis experienced permanent menopause, while >50% of patients under age 40 had resumption of menses [42]. Similar rates of amenorrhea were seen in a study of 249 women receiving epirubicin [43].

Taxanes have been shown to improve survival in the adjuvant setting in patients with node-positive breast cancer and are increasingly used in high risk node-negative patients, particularly young women, as young age appears to be a significant risk factor for recurrent disease. The true impact of taxanes on menstrual function is difficult to determine, as they are most often given in sequence or combination with anthracycline-based therapy. Although several small trials have reported similar or decreased rates of amenorrhea with the addition of taxane to anthracyline-based chemotherapy, a number of trials report higher

rates of amenorrhea in patients who receive both classes of drugs [33]. For example, in the BCIRG-001 study, in which 1491 patients were randomized to either docetaxel, doxorubicin and cyclophosphamide (TAC) or fluorouracil, doxorubicin and cyclophosphamide (FAC), the rate of amenorrhea was 10% higher in patients receiving the taxane. Similar results were seen in a smaller survey study comparing rates of amenorrhea in women who received four cycles of doxorubicin and cyclophosphamide (AC) and those who received AC followed by taxane [44]. Although there are little data comparing the effect of docetaxel with that of paclitaxel in terms of menstrual function, one observational study suggested higher initial rates of amenorrhea with docetaxel versus paclitaxel, but rates were equivalent at 3 years after treatment [40]. Interestingly, in that study, women receiving AC alone or with taxane had initially higher rates of amenorrhea than those receiving CMF, but there was significant recovery of menstrual function after anthracycline-based therapy, while CMF resulted in a continued steady decline in the proportion of patients with menstrual bleeding, suggesting that the effect of CMF on the ovaries is more likely to be permanent.

## Endocrine therapy and reproductive function

About 60% of premenopausal patients have hormone receptor-positive breast cancer, and most will be offered endocrine therapy, either alone or in combination with chemotherapy. The mainstay of hormonal therapy in premenopausal women is treatment with tamoxifen for a period of 5 years, which has been shown to improve both recurrence-free and overall survival in this population [37]. In one study in which women were randomized to receive or not receive tamoxifen along with chemotherapy, the use of tamoxifen decreased the likelihood of menstrual cycling at 1 and 2 years, regardless of chemotherapy regimen [40], and some women developed irregular menses on tamoxifen. As noted above, tamoxifen may have teratogenic effects and thus should not be used during pregnancy. In premenopausal women, treatment with a GnRH may also be considered, as some data suggest that these agents may improve outcomes when used in addition to, or in lieu of, tamoxifen and/or chemotherapy [37]. While it is commonly assumed that only chemotherapy affects fertility, it is important to note that hormonal therapy itself and the delay of conception in order to receive several years of hormonal therapy may also affect a woman's chance of successful conception. To date, no randomized studies have examined the effect of hormonal therapy on pregnancy outcome [45]. Given the lack of data, many patients with hormone receptor-positive breast cancer who desire pregnancy, and their treating physicians, struggle with whether to shorten the duration of endocrine therapy or to temporarily interrupt endocrine therapy in order to achieve pregnancy.

# Fertility preservation in breast cancer patients

As a result of higher survival rates among women treated for breast cancer, there is an increasing emphasis on quality of life among survivors, and fertility preservation is a key issue among young women undergoing therapy for breast cancer. The American Society of Clinical Oncology guidelines recommend that all patients interested in future fertility should be referred for consideration of fertility preservation [46]. Data suggest that although cancer survivors can become parents through third-party reproduction (such as gamete donation and adoption), most would prefer to have biological offspring [47]. Fertility preservation options in women with breast cancer depend on the patient's age, type of treatment planned, whether or not she has a partner and the time available prior to starting therapy.

## Embryo cryopreservation

To date, embryo cryopreservation is considered the more effective approach to fertility preservation. It has been routinely used for storage of surplus embryos after in vitro fertilization treatment for infertility. The post-thaw survival rate of embryos is in the range of 35–90%. Successful implantation rates range from 35 to 90%, and if multiple embryos are stored, cumulative pregnancy rates can be >60% [48]. This approach requires either a male partner or sperm donor. Typically, the patient undergoes 2 weeks of ovarian stimulation with daily injections of follicle stimulating hormone (FSH) beginning at the onset of menses. Follicle development is monitored by serial ultrasound imaging and blood tests. At the appropriate time, human chorionic gonadotropin is administered to stimulate ovulation and oocytes are collected using transvaginal aspiration under ultrasound guidance. Oocytes are

then fertilized in vitro and cryopreserved. In women with hormone receptor-positive breast cancer (the majority of women diagnosed with breast cancer), concerns exist about possible detrimental effects of the use of endocrine agents to stimulate ovulation on breast cancer outcome. Oocyte collection without ovarian stimulation can be attempted, but the embryo yield is very low. Alternative hormonal stimulation approaches, such as the use of letrozole or tamoxifen concurrent with FSH, have been attempted, and do not appear to increase cancer recurrence rates [49]. Even in women with hormone receptor-negative breast cancer, concerns about delays in initiation of cancer treatment may limit the feasibility of this approach. Because the process of ovarian stimulation must begin at the time of menses and then takes 2 weeks, chemotherapy and other life-saving treatments may be delayed. While data suggest that the hormonal milieu of natural pregnancy does not adversely affect breast cancer outcomes, there are no data regarding safety of hormones to support pregnancy (such as high dose progesterone) after embryo implantation. In studies published to date addressing survival of breast cancer patients who have become pregnant after diagnosis and treatment, only a small percentage of included patients conceived via assisted reproductive technologies [50].

## Oocyte preservation

Cryopreservation of unfertilized oocytes is an option for women for whom a partner is unavailable. The ovarian stimulation and harvesting process is identical to that used for embryo cryopreservation, but the oocytes are stored unfertilized and then subsequently thawed and fertilized in vitro. Thus, this technique is associated with the same issues regarding timing, potential delay of chemotherapy and exposure to hormones. Unfortunately, oocyte freezing is technically more complex than embryo cryopreservation and unfertilized oocytes are more prone to damage during cryopreservation; thus, the overall successful pregnancy rate is likely lower with this procedure [51].

## Ovarian tissue cryopreservation

The process of ovarian tissue cryopreservation involves freezing thin slices of the ovarian cortex, which contains a rich reserve of primordial follicles. This investigational method of fertility preservation requires neither a sperm donor/partner nor ovarian stimulation. The first ovarian transplant procedure was reported in 2000 [52], and since then there have been several case reports of successful pregnancies resulting from these procedures [53–55]. Ovarian tissue is removed laparoscopically (this procedure requires general anesthesia) and frozen, and can be thawed and re-implanted at a later date, either orthotopically (in the pelvis) or heterotopically (in subcutaneous tissue in the forearm or abdomen); studies have reported restoration of ovarian function with both methods. Although the cryopreservation technique is highly effective, a large number of follicles may be lost due to ischemia at the time of re-implantation, so typically the cortex from an entire ovary is used.

One hypothetical concern with this method is the potential for re-introduction of metastatic cancer cells. However, in breast cancer patients without evidence of systemic disease, the likelihood of occult ovarian metastases appears to be extremely low, and in the limited reported literature, no cases of cancer recurrence after ovarian transplantation have been noted [46].

## Donor eggs and surrogacy

In patients in whom oocyte or embryo cryopreservation prior to treatment was not possible, particularly those with low ovarian reserve or premature menopause, in vitro fertilization using donor ova may be an option. One advantage to this method is a higher success rate using fresh rather than frozen ova. With appropriate egg donors, success rates may exceed 60% per embryo transfer [56]. For those with a high risk of recurrence, or those on long-term therapy with tamoxifen or aromatase inhibitors, gestational surrogacy may be a viable alternative to pregnancy.

## Attempts to preserve fertility during chemotherapy: suppression of ovarian function

Ovarian suppression with a GnRH agonist during chemotherapy treatment has been suggested as a means to preserve long-term menstrual function, though this strategy is controversial. A small study comparing 54 patients with retrospective controls suggested a benefit in preservation of ovarian function among women undergoing chemotherapy for Hodgkin's and non-Hodgkin's lymphoma [57], with 93.7% of those receiving GnRH agonists resuming menses vs 37% of historical controls. In a second study examining the use of GnRH agents for ovarian

function preservation in patients receiving chemotherapy ($n = 62$) compared with retrospective controls ($n = 55$), the percentage of patients resuming ovulation and menses was significantly higher in the GnRH group [58]. In the first prospective randomized trial of this strategy, 17 women were randomized to either buserelin or control prior to and during chemotherapy; at 3 years, 4 of 8 women receiving buserelin and 6 of 9 controls were amenorrheic. In a more recent randomized study that included 80 women receiving chemotherapy for breast cancer, the addition of GnRH agonist prior to and during chemotherapy significantly improved the rate of resumption of menses (89.6% versus 33.3%) and spontaneous ovulation (69.2% versus 29.6%) [59]. Though compelling, these data require confirmation; the Southwest Oncology Group (SWOG) is currently conducting SWOG S0230 – a randomized trial with an accrual goal of 458 patients evaluating GnRH agonists for ovarian function preservation in women with hormone receptor-negative breast cancer who receive chemotherapy [46].

## Contraception following breast cancer

As many chemotherapeutic and hormonal agents used for treatment of breast cancer are known or suspected to be teratogenic, pregnancy should be avoided during active treatment of breast cancer. Hormonal contraceptives are generally felt to be contraindicated, particularly in women with estrogen and progesterone receptor-positive tumors, although there is little established evidence addressing their use [60]. Recent small studies have addressed the use of an intrauterine levonorgestrel–releasing system (Mirena), which delivers high local but low systemic doses of progesterone, and suggest that in addition to providing effective contraception, this device may lower the risk of endometrial pathology in tamoxifen users (61]. However, there is limited data on the effect of Mirena on cancer incidence and recurrence. Subgroup analyses from one recent cohort study suggested a trend toward increased risk of recurrence among women using Mirena at the time of diagnosis who continued with the device *in situ*, but this effect was not seen among women with insertion of the device after a breast cancer diagnosis [62]. In the absence of solid data, non-hormonal contraceptive methods remain the standard for women with a previous diagnosis of breast cancer.

## Pregnancy following breast cancer

Based on limited retrospective data, pregnancy does not appear to compromise the survival of women with a history of breast cancer, and no deleterious effects have been demonstrated in the fetus [63]. The rate of pregnancy among women of reproductive age with a diagnosis of cancer is estimated to be about 50% lower that of age-matched peers [64]. One retrospective study performed by the International Breast Cancer Study Group described a series of 94 patients who became pregnant after a diagnosis of breast cancer [65]. Seventeen percent of study participants had their first subsequent pregnancy within 1 year of their breast cancer diagnosis, 24% between 1 and 2 years, 23% between 2 and 3 years and 31% achieved pregnancy 3 or more years after diagnosis. This study also compared the prognosis among these women to that of 188 matched controls, and found that those women who attained pregnancy had superior survival compared with controls, although this difference may represent a bias toward women with good prognosis tumors and better overall health being more likely to seek and achieve childbearing. In any case, this and other studies do not indicate a detrimental effect of subsequent pregnancy on survival, especially if some interval occurs between the cancer and the pregnancy [50, 65–68]. Some physicians recommend that patients wait a couple of years after diagnosis before attempting conception. This allows both time for the body to heal and time for early recurrences of cancer to manifest, which may influence the decision to become a parent [50, 69]. In one recent study evaluating pregnancy and disease outcomes based on time from diagnosis to conception, women who conceived 24 or more months after diagnosis had significantly improved survival compared with those who did not conceive; women who conceived 6–24 months after diagnosis also showed a trend toward improved survival, though this was not statistically significant [50]. Given these results, coupled with the fact that fertility rates decline significantly with age, the authors suggested that it may not be necessary to advise waiting more than 6 months to attempt conception in patients with localized disease.

The risk of relapse and time to recurrence of breast cancer is associated with many factors, including stage at diagnosis, lymph node status, tumor grade and hormone receptor status. Notably, recurrence risk for hormone receptor-negative tumors is greatest in the first 2 years after diagnosis, and then drops

significantly [70]. Annual risk of recurrence for hormone receptor-positive tumors is more stable, and is relatively constant from years 1–5 and then drops slightly but stays constant from years 5–12. As younger women are more likely to develop hormone receptor-negative disease, these risks must be considered when making decisions regarding pregnancy. With regard to hormone-receptor positive disease, it is important to note that the currently recommended duration of adjuvant endocrine therapy for premenopausal women is 5 years, and the decision to attempt conception necessitates discontinuation of endocrine therapy. Thus, the decision regarding when to attempt conception should be individualized.

## Lactation following breast cancer

Observational data suggest that breastfeeding after a diagnosis of breast cancer does not affect prognosis, and there are no data to suggest that breastfeeding poses any health risk to the child [71]. Women who have been treated with breast-conserving surgery will require radiation to the affected breast, which reduces the success of breastfeeding on that side. In one small study of 11 patients with 13 pregnancies, successful lactation in the treated breast occurred in four instances, and one patient was able to breastfeed from the treated breast for 4 months [72]. Time from treatment to lactation did not appear to have an effect on lactation success. However, circumareolar incisions did appear to adversely affect ability to breastfeed.

## Summary and conclusions

Breast cancer is a common disease among women and frequently affects premenopausal women. Breast cancer during pregnancy is rare and poses unique issues relating to ensuring adequate treatment of the patient while not endangering the fetus. Additionally, as the mean age of childbearing increases, fertility issues after diagnosis and treatment of breast cancer are increasingly relevant, and present significant challenges to patients and their healthcare providers. Treatment for breast cancer, particularly chemotherapy, can have permanent effects on reproductive capability. While embryo cryopreservation, oocyte preservation and ovarian tissue cryopreservation are among the options available to increase future fertility in young women at the time of diagnosis of breast cancer, the hormonal manipulation and timing requirements of these procedures currently limit the feasibility for many breast cancer patients. Further research is needed to determine which modalities can successfully aid in protecting and aiding reproductive potential in women undergoing breast cancer therapy who desire future fertility.

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Chapter 7

# Pediatric cancer therapy and fertility

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## Background

There are currently an estimated 270 000 survivors of childhood cancer in the USA [1]. The 5-year survival rate for all childhood cancers is approximately 80% [2], but survival is often associated with a cost. Treatment with past and contemporary regimens of chemotherapy and radiation can affect future fertility. Sterility, infertility or subfertility can result from gonadal removal or damage to germ cells from adjuvant therapy. Damage to the gonads by irradiation or chemotherapy depends upon the cancer survivor's gender, age at time of treatment, dose of radiation and fractionation schedule, and total dose and nature of chemotherapy given [3, 4]. This chapter reviews pediatric cancer therapy and its consequences on fertility.

## Effects of chemotherapy and radiation on ovaries

### Normal physiology and potential for fertility

The outer cortex of the ovary contains oocytes and is the site of hormone production in females. Female oocyte production ceases during fetal development, and girls are therefore born with a finite number of oocytes (2 million at birth with approximately 300 000 left at puberty) [5]. The normal physiology of oocyte maturation begins in utero, occurs continuously and is initially gonadotropin-independent. At puberty, the gonadotropin-dependent phase begins, and follicles are primed by follicle stimulating hormone resulting in granulosa cell proliferation. Luteinizing hormone then triggers ovulation with a potential for fertilization [6]. The normal premenopausal ovary contains degenerating ova and follicles in varying stages of maturity. A typical female will release 300–500 mature eggs during her reproductive life span.

Due to the non-renewable nature of a female's oocytes, the oocytes are quite susceptible to damage [7]. With the depletion of oocytes by radiotherapy, chemotherapy or normal senescence, the ovaries undergo atresia [6]. Alkylating agents are the most common chemotherapeutic agents associated with gonadal damage; these agents are not cell-cycle-specific and thus do not require cell proliferation for their cytotoxic actions; however, dividing cells are more susceptible to damage [8, 9]. As a result, menstruation and estrogen production ceases and menopause occurs. Ovarian hormones have critical physiological effects on other organs and bodily processes including the stimulation of libido, the maturation and function of breasts and vagina, bone mineralization and the integrity of the cardiovascular system.

### Effects of chemotherapy

Chemotherapy can cause infertility, premature ovarian failure, menstrual irregularity and delayed puberty. The effects of chemotherapy on ovarian function are both agent and dose-dependent, and this effect may be additive to that resulting from abdominopelvic radiotherapy. Alkylating agents such as cyclophosphamide affect the resting oocyte in a dose-dependent, cell cycle-independent manner [10] by affecting undeveloped oocytes and possibly pre-granulosa cells of primordial follicles [11]. The results of alkylating agents are more pronounced in post-pubertal as compared to pre-pubertal females, due to the fact that post-pubertal females have fewer remaining oocytes. Risks of menstrual irregularity, ovarian failure and infertility increase with age at treatment, as the normal aging process is accompanied by an ongoing depletion of oocytes. Amenorrhea and premature ovarian failure occur more commonly in adult women treated

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**Table 7.1** Gonadotoxic chemotherapeutic agents

| |
|---|
| Procarbazine |
| Cyclophosphamide |
| Chlorambucil |
| Mustine |
| Melphalan |
| Busulfan |
| Nitrosoureas (BCNU and CCNU) |
| Ifosfamide |
| Cytosine arabinoside |
| Cisplatin |

BCNU, 1,3-*bis*(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

with cyclophosphamide than in adolescents [12, 13]. Younger females can tolerate higher doses of alkylating agents without impairment of fertility when compared to adult females [10, 14–16].

In a study examining the effects of adjuvant chemotherapy on women undergoing treatment for soft tissue sarcoma, authors noted that chemotherapy with doxorubicin, cyclophosphamide and high dose methotrexate produced irregular menses in 20% of women and persistent amenorrhea in 20% of women [17]. Other chemotherapy agents that have been associated with ovarian damage include procarbazine [18]. Table 7.1 lists common chemotherapy agents that are known to be gonadotoxic.

Frequently, there may initial evidence of ovarian failure, but recovery often occurs. In a study by Sanders *et al.*, women who received high dose cyclophosphamide (50 mg/kg/day for 4 days) prior to bone marrow transplantation for aplastic anemia all developed amenorrhea following transplantation. Fortunately, >80% of the survivors had recovery of normal ovarian function between 3 and 42 months after transplantation [19]. Ovarian function was evaluated in women treated with different drug combinations by Green *et al*; in women who received a low dose cyclophosphamide-containing drug combination for non-Hodgkin's lymphoma, ovarian function was normal in all women; however, women who received a combination of whole abdominal radiation with a combination of low dose alkylating agents had ovarian failure [20].

Chemotherapy regimens used as conditioning for stem cell transplantation are highly gonadotoxic. Recovery of ovarian function is rare following regimens that include busulfan and cyclophosphamide, though melphalan-based regimens show less reproductive toxicity [21].

In addition to the risk of infertility, female survivors of childhood cancer are also at risk for premature ovarian failure (early menopause) [22]. Byrne *et al.* reported a ninefold increase in the incidence of menopause during their early 20s in female survivors of childhood cancer when compared to the general population. This study also examined women who reported having normal menses after the completion of chemotherapy and noted that 42% of women reported premature menopause (ovarian failure by the age of 31) compared to 5% of the general population [22]. Thus, the presence of apparently normal ovarian function at the completion of chemotherapy should not be interpreted as evidence of lack of injury to ovarian tissue. Studies have also reported disturbances in pubertal progression in approximately 30% of patients treated with alkylating agents during puberty [10, 23, 24]. The Childhood Cancer Survivor Study reported that 8% of female childhood cancer survivors experienced a non-surgical premature menopause compared to 0.8% of sibling controls [25].

## Effects of radiation

Abdominal, pelvic and total body irradiation (TBI) may result in ovarian and uterine damage. Damage induced by radiotherapy results in progressive and irreversible damage in the ovary, causing amenorrhea and infertility. The oocyte is very sensitive to radiation and undergoes irreversible damage at 2 Gy in 50% of patients [26]. Radiation causes a decrease in the number of ovarian follicles, impaired follicular maturation, cortical fibrosis and atrophy, generalized hypoplasia and hyalinization of the capsule.

The frequency of ovarian failure following abdominal radiotherapy is related to the age at time of irradiation and the radiation therapy dose received by the ovaries. Females treated prior to the onset of puberty have a greater number of ova than older females; thus ovarian function is more likely to be preserved after radiotherapy in pre-pubertal females compared to post-pubertal females [27, 28]. Whole abdominal radiotherapy produces severe ovarian damage. Wallace *et al.* demonstrated that >25% of female childhood cancer survivors who received abdominal irradiation therapy doses of 2000–3000 cGy had premature menopause before the age of 16 years [29].

**Table 7.2** Effect of fractionated ovarian X-irradiation on ovarian function in women of reproductive age irradiated for malignant or non-malignant disease

| Minimum ovarian dose (Gy) | Effect |
|---|---|
| 0.6 | None |
| 1.5 | No deleterious effect in most young women. Some risk of sterilization especially in women aged >40 years |
| 2.5–5.0 | Variable. Aged 15–40 years: about 60% sterilized permanently, some with temporary amenorrhea. Aged >40 years; usually 100% permanent sterilization |
| 5–8 | Variable. Aged 15–40 years: about 70% sterilized permanently; of the remainder, some with temporary amenorrhea |
| >8 | 100% permanently sterilized |

Adapted from Ash [36].

Other reports of childhood survivors treated with whole abdomen radiotherapy and craniospinal radiotherapy reveal similar results [30–32].

All women who receive TBI prior to stem cell transplantation develop amenorrhea [33] immediately after treatment. Fortunately, some may have recovery of normal ovarian function. Sanders *et al.* examined ovarian function following bone marrow transplantation for aplastic anemia or leukemia and noted that <6% of survivors had recovery of normal ovarian function; these results also indicated that return of normal ovarian function was highly correlated with age <25 years [19]. Recovery of ovarian function among female childhood cancer survivors who received TBI is more favorable if the TBI was given in fractions and if the child was pre-pubertal [21].

Ovarian failure following radiotherapy is also correlated with the treatment volume. In a study looking at ovarian failure in long-term survivors of childhood malignancy [34], ovarian failure occurred in almost 70% of women who received radiation therapy that included their ovaries in contrast to 0% of women whose irradiation field did not include their ovaries. It is also important to consider the risk of ovarian failure related to radiotherapy when fields outside the abdomen and pelvis have been irradiated. Direct or scattered irradiation from craniospinal radiotherapy has been shown to affect ovarian function [33, 35]. Table 7.2 shows the effect of differing minimum doses of radiation on the ovaries [36].

# Effects of chemotherapy and radiation on testes

## Normal physiology and potential for fertility

Male germinal stem cells are present from the time of birth, but they do not develop into gametes capable of fertilizing an oocyte until a male goes through puberty. The testis is composed of cells that aid in the development of sperm; Sertoli cells support and nurture developing germ cells and are also the site of production of the glycoprotein hormone, inhibin; and Leydig cells are responsible for testosterone synthesis [3]. Spermatogenesis is a process that begins at puberty and continues throughout life. There is a steady turnover of germ cells in the pre-pubertal testis that undergo spontaneous degeneration before a haploid state of maturation is reached. Studies have hypothesized that during this steady state of turnover, cytotoxic therapy affects fertility. Testicular function may be damaged by surgery, irradiation and/or chemotherapy.

## Effects of chemotherapy

Testicular dysfunction is among the most common long-term side effect of chemotherapy in men. The germinal epithelium is very susceptible to injury by cytotoxic drugs secondary to its high mitotic rate. Compared with the germinal epithelium, Leydig cells are relatively resistant to the effects of chemotherapy [37–39]. However, with more intensive gonadotoxic regimens, reductions in testosterone concentrations have been noted that may be clinically relevant.

Similar to females, regimens that include high doses of alkylating agents are the most toxic to gonadal tissue. Total dose of cyclophosphamide is one factor that influences the severity of testicular damage. In survivors of Ewing's and soft tissue sarcoma, who received a cumulative dose of cyclophosphamide >7.5 g/m$^2$, there was an increased risk of persistent oligo- or azoospermia [40]. In studies of male childhood survivors of acute lymphoblastic leukemia (ALL), Blatt *et al.* reported normal testicular function in boys treated for ALL with therapy that did not include cyclophosphamide or intravenous cytosine arabinoside [41]. Drugs such as procarbazine, cyclophosphamide and chlorambucil have been shown to produce prolonged azoospermia in >90% of men [42–44]. Combination chemotherapy that includes an

alkylating agent and procarbazine causes severe damage to the testicular germinal epithelium [42, 44–46].

Studies of adolescent and young adult male survivors of Hodgkin's lymphoma have shown that both the chemotherapeutic regimen and dose intensity are important variables affecting reproductive potential [4, 44, 47]. Those treated with 6 cycles of chemotherapy that included nitrogen mustard, vincristine, prednisone and procarbazine had >90% of infertility due to azoospermia [44, 47]. However, the adolescent and young adult males who received 3 or fewer cycles of identical therapy had only a 50% rate of infertility [47]. In a study examining more contemporary therapy in Hodgkin's lymphoma, authors found that patients treated with a regimen of adriamycin, bleomycin, vinblastine and dacarbazine had a 33% risk of infertility [4]. In a recent cohort study conducted by the European Organization for Research and Treatment of Cancer, exposure to alkylating chemotherapy was associated with a significantly higher risk of gonadal dysfunction among male patients and longer recovery time of gonadal function [48].

Chemotherapy that includes platinum compounds can cause prolonged azoospermia in up to 50% of men [49]. Conditioning regimens that include high doses of alkylating agents and TBI used for stem cell transplantation cause prolonged azoospermia in more than 50% of survivors [50, 51].

Chemotherapy appears to lower healthy sperm counts in cancer survivors but, after an adequate time off of therapy, some studies suggest that DNA integrity of sperm is re-established similar to age-matched controls; the amount of time off therapy required for this recovery has not been adequately quantified [52].

## Effects of radiation

When testes are exposed to radiation, sperm count begins to decrease. Dependent upon dosage, temporary or permanent sterility may result [53]. The degree and permenancy of radiotherapy-induced testicular damage also depends upon the treatment field and fractionation schedule. Table 7.3 shows the effects of fractionated radiation on spermatogenesis and Leydig cell function [54]. Males who receive radiation to the abdominal or pelvic region may still regain partial or full sperm production depending upon the amount of injury to the testes. Unlike the germinal epithelium, Leydig cell function may be more prone to damage from irradiation in pre-pubertal life than adulthood [55]. Testicular radiation with doses >20 Gy is associated with Leydig cell dysfunction in pre-pubertal boys, while Leydig cell function is usually preserved with doses of as much as 30 Gy in sexually mature males. Exposing the testes to ionizing radiation at a dose <6 Gy causes disturbances of spermatogenesis and altered spermatocytes with recovery periods dependent on dose [54]. Doses >6 Gy cause permanent infertility by destroying all stem cells [56].

The testes are directly irradiated in situations such as testicular relapse in ALL, and the high doses of radiotherapy required (often 2400 cGy) results in both sterilization and Leydig cell dysfunction [57]. Total body irradiation used for stem cell transplantation conditioning can cause permanent gonadal failure in approximately 80% of males [21]. Craniospinal irradiation produced primary germ cell damage in almost 20% of children with ALL in a study that utilized the Childhood Cancer Survivor Study [58]. This study demonstrated the testes sometimes receive radiation via body scatter. Scatter occurs when X-rays interact with tissues near the target of interest, resulting in secondary X-rays that then hit the target [59]. The amount of scattered radiation is a function of the proximity of the radiation field to the target, the field size and shape, the X-ray energy and the depth of the target. Of these, distance from the field edge is the most important factor. Scatter dose to the testes may be an issue when treating a field that extends into the pelvis.

## Risk of infertility after treatment

Studies have demonstrated that when evaluated as a group, the fertility of childhood cancer survivors is impaired. A multicenter study examining 5-year childhood and adolescent survivors of solid tumor cancers and Hodgkin's lymphoma demonstrated a 15% incidence in impaired fertility in survivors; males having more problems than females [60]. Other studies have found a number of variables associated with decreased fertility after cancer treatment in childhood and adolescent survivors of cancer and bone marrow transplantations. These variables include: older age at time of cancer therapy, type of therapy, site of therapy and gender [61–65].

It is important for physicians treating children and adolescents to recognize the risks associated with radiation and chemotherapy and the effects treatment will have on future fertility. In 2006, the American Society of Clinical Oncology provided recommendations

**Table 7.3** The effects of fractionated radiation on spermatogenesis and Leydig cell function

| Testicular dose (cGy) | Effect on spermatogenesis | Effect on Leydig cell function |
|---|---|---|
| <10 | No effect | No effect |
| 10–30 | Temporary oligospermia | No effect |
| 30–50 | Temporary oligospermia 4–12 months after radiation: 100% recovery by 48 months | No effect |
| 50–100 | 100% temporary azoospermia 3–17 months after radiation. Recovery begins at 8–26 months | No effect |
| 100–200 | 100% azoospermia 2–9 months after radiation. Recovery begins at 11–20 months | No change in testosterone |
| 200–300 | 100% azoospermia beginning at 1–2 months. May lead to permanent azoospermia. Recovery variable generally years) | No change in testosterone |
| 1200 | Permanent azoospermia | Decreased basal testosterone. Replacement hormone not needed to ensure initiation of puberty |
| 2400 | Permanent azoospermia | Decreased testosterone. Replacement needed to ensure puberty |

Adapted from Schwartz [54].

for oncologists to help address the possibility of infertility with patients treated during their reproductive years [66]. The guidelines state that oncologists should be prepared to discuss fertility preservation options or to refer the patient to reproductive specialists for further information prior to initiation of therapy. However, there is no consensus on when the appropriate age of reproductive potential actually occurs or at what age patients should be referred to a reproductive specialist.

## Preservation of fertility

The options for fertility preservation before treatment differ between females and males. Males have more available options that are less invasive, less expensive and more effective.

### Preventive strategies for females before treatment

Progress in reproductive endocrinology has resulted in the availability of several potential options for preserving or permitting fertility in females prior to receipt of potentially toxic chemotherapy or radiotherapy. These procedures are described elsewhere in this book; we will discuss some strategies specific to pediatric cancer survivors.

In vitro fertilization and subsequent embryo cryopreservation has been successful and is an established technique [9]; this is the only non-experimental pretreatment fertility preservation option for adolescent and young adult women. This intervention is technically complex and can delay initiation of cancer therapy by 2–4 weeks. This intervention is also limited to females who are either involved in a stable relationship or willing to identify a known or anonymous sperm donor. These issues often preclude this intervention as an option for adolescent females. Also, this technique requires ovarian stimulation, which precludes it being an option for those females with estrogen-sensitive tumors.

Ovarian tissue cryopreservation is a process in which normal, functioning ovarian tissue is excised from the ovary and stored cryogenically and is the only option that can be offered to pre-pubertal girls [67]. Currently this technique is available only in certain parts of the USA as an experimental protocol. This technique involves obtaining primordial follicles from a laprascopic biopsy. The immature follicles are smaller than mature oocytes and more tolerant to freezing and thawing [6]. Hundreds of immature oocytes are cyropreserved without the necessity of ovarian stimulation and subsequent delay of cancer treatment. Ideally, the stored ovarian tissue is thawed and autotransplanted into the donor once treatment has been completed. Studies in humans are still in their infancy, though there are scattered published case reports that demonstrate its efficacy; a recent paper details 8 years of

experience in adult female cancer patients with positive results [68, 69]. Kim *et al.* reported on four young cancer survivors who had their ovaries removed prior to the initiation of cancer therapy and then had a heterotopic autotransplantation of their ovarian tissue over the course of a few years after completing therapy. Their report concluded that ovarian function was re-established in all three patients [69].

## Preservation of fertility during treatment for females

Reduction in the dose or use of alkylating agents and abdominopelvic radiotherapy is the most effective means of preserving ovarian function and promoting positive reproductive outcomes in pediatric cancer survivors. Studies have shown that movement of the ovaries out of the field of radiation (ovariopexy), either laterally, toward the iliac crest or behind the uterus may help preserve fertility when high doses of radiation therapy are used [70]. By relocating the ovaries laterally, it is possible to shield them during radiation of the para-aortic and femoral lymph nodes. This may also be helpful for young girls or adolescents undergoing craniospinal radiotherapy for brain tumors. The ovaries should be marked by the surgeon with clips that can later be identified by simulator film. Central pelvic blocking at the time of "inverted Y" field will prevent direct irradiation, though scatter dose and transmitted dose will be inevitable [53]. Pelvic radiation, however, still provokes an irradiation of the ovary of 5–10%, even if the ovaries are transposed outside the irradiation area [71]. Although ovarian transposition is relatively effective at preserving the endocrine function of the ovary in approximately 85% [72] of cases, 1 study demonstrated that only approximately 15% of patients who wish to become pregnant achieve this goal [70].

## Preventive strategies for males before treatment

Sperm cryopreservation after masturbation is the most established and effective method of fertility preservation in males [66]. Sperm should be collected before initiation of chemotherapy or radiotherapy. Studies have demonstrated that sperm quality may be compromised in males with Hodgkin's lymphoma, leukemia and testicular cancer [73]. These limitations have been largely overcome by improving in vitro fertilization technology. Intracytoplasmic sperm injection allows successful fertilization with a single sperm. Collection of semen through masturbation in adolescents and young males may be compromised by embarrassment and issues with informed consent. Alternative methods of obtaining sperm include testicular aspiration or extraction, electroejaculation under sedation or anesthesia or from a post-masturbation urine sample [74, 75] Pre-pubertal males pose a challenge for fertility preservation. Currently, testicular tissue cryopreservation is an experimental option. Ginsberg *et al.* reported that 76% of 21 families of pre-pubertal boys with newly diagnosed malignancies consented to testicular biopsy cryopreservation and that none of these patients had any postoperative sequelae [76].

## Preservation of fertility in males during treatment

Cryopreservation of sperm has become the standard practice and should be offered to all newly diagnosed, post-pubertal males at risk for infertility. Gonad shielding can be used during radiation therapy but is only possible with selected radiation fields and anatomy [66]. Fraass *et al.* reported that a gonadal shield forming a cup around the testes to reduce the testicular dose [45] led to a 3–10-fold reduction in the radiation dose to the testes, depending upon the distance from the proximal edge of the field. In almost all cases, the measured dose to the testes was <1% of the prescription dose. Therefore, for a patient receiving 5000 cGy to a pelvic field, the dose to the testes would be <50 cGy, which would prevent permanent azoospermia.

# Emerging data on fertility after cancer treatment

Compared to healthy siblings, the fertility of childhood and adolescent cancer survivors is impaired. The most significant differences in the relative fertility rates occur in male survivors treated with an alkylating agent with or without irradiation [51, 60, 64].

Fertility may be impaired by factors other than the absence of sperm or ova. Anatomic changes due to surgery and radiation can affect fertility as well. Retrograde ejaculation occurs with a significant number of men who undergo retroperitoneal lymph node dissection for testicular cancer. Uterine structure may be affected by abdominal irradiation in females.

Another issue that is important to disclose to families is the cost of fertility preservation. Patients and families are usually responsible for the costs of preservation, as most insurance companies do not cover these costs. Fallat and Hutter reported that the cost of sperm cryopreservation after masturbation was approximately $1500 for 3 samples stored for 3 years in 2006 [77]. This cost was exponentially higher if alternative methods were needed to obtain sperm or for prolonged storage [66]. Unfortunately, the cost for fertility preservation for females, which involves more procedures and anesthesia, can easily be more than $10 000.

## Role of physician

Patients who will receive therapy with the potential to limit or abolish fertility need sensitive, informed management. Important aspects of management have been discussed in the previous sections and involve considerations of gonadal protection, germ cell storage and assisted fertilization. It is imperative that physicians present this information to families and patients prior to the initiation of chemotherapy or radiotherapy. A recent study reported that both pediatric and adult oncologists are uncomfortable with discussing fertility preservation with cancer patients. Quinn *et al.* reported that a physician's discomfort with this topic was due to a number of variables that included language barriers, lack of knowledge and success rates of fertility preservation techniques available and perception that the subject of fertility preservation adds more stress [78]. It is important for oncologists to have access to improved training that will remove these biases and facilitate these discussions. A physician's encouragement is a strong predictor of whether an optional intervention will be considered by a patient [77]. Oncologists have a responsibility to inform parents and age-appropriate patients about the likelihood that their cancer treatment may permanently affect their fertility [66]. Parents of minors or age-appropriate children should be informed of their prognosis in realistic terms. The success rates, costs and experimental nature of specific assisted reproduction techniques should be discussed. Suggestions have been previously published regarding counseling parents and patients about the preservation of fertility in children and adolescents with cancer. The authors recommended that evaluation of candidacy for fertility preservation should involve a team of specialists that includes a pediatric oncologist, a radiation oncologist, a fertility specialist, an ethicist and a mental health professional [77]. Table 7.4 lists the guidelines [77].

**Table 7.4** Guidelines for parents and patients about preservation of fertility

| | |
|---|---|
| 1 | Offer cryopreservation of sperm whenever possible to male patients and families |
| 2 | Discuss current fertility preservation options for female children and adolescents |
| 3 | When considering actions to preserve a child's fertility, parents need to consider child's assent, procedures involved and whether procedures are proven or experimental |
| 4 | Instructions concerning disposition of stored gametes, embryos or gonadal tissue in the event of patient's death should be legally outlined |
| 5 | Concerns about welfare of resulting offspring of childhood cancer survivor should not be cause for denying reproductive assistance to patient |

Adapted from Fallat [77].

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Chapter 8

# Cancer epidemiology and environmental factors in children, adolescents and young adults

Karina Braga Ribeiro and Paolo Boffetta

## Introduction

Cancer is a rare disease in childhood. In developed countries, only about 0.5% of all cases occur in children under 15 years of age. The incidence rates range between 96 and 138/million children per year for males and between 70 and 116/million children for females [1]. Contrary to what is observed in adults, epithelial cancers (carcinomas) are rare among children, and in this age group solid tumors are predominantly embryonal. Therefore, it is more appropriate to classify childhood cancers according to their histology. According to the International Classification of Childhood Cancer [2], childhood tumors are classified into 12 major diagnostic groups: leukemias, lymphomas, central nervous system (CNS) tumors, sympathetic nervous system tumors, retinoblastomas, renal tumors, liver tumors, bone tumors, soft tissue sarcomas, germ cell tumors, epithelial tumors and other and unspecified malignant cancers (Table 8.1 [2]). Leukemias, brain tumors and non-Hodgkin's lymphomas (NHLs) are the most frequent pediatric cancers in developed countries, representing almost 60% of all cases, while in developing countries NHLs are more common than brain tumors (Figure 8.1) [3]. Each year, an estimated 160 000 cancers are diagnosed in children worldwide, and an additional 240 000 cancers in adolescents and young adults (aged 15–24 years) [1].

## Descriptive epidemiology

### Childhood cancer

According to the last edition of *Cancer Incidence in Five Continents*, the highest cancer incidence rates, for all tumor sites except skin, are observed in Kuwait (non-Kuwaitis, 418/million), Italy (Brescia and Naples, 250 and 234 new cases/million, respectively), Brazil (São Paulo and Brasília, 224 and 219/million, respectively), Switzerland (Vaud, 214/million), Croatia (203/million) and the USA (non-Hispanic white population, 179/million) for males, while for females high incidence is noted in Kuwait (non-Kuwaitis, 500 new cases/million), Brazil (Brasilia and São Paulo, 195 and 190/million, respectively), Cyprus (190/million), Italy (Salerno, 172/million), Germany (Munster, 168/million), Portugal (southern region, 160/million) and the USA (non-Hispanic white population, 155/million) (Figure 8.2a, b) [1]. On the other hand, mortality rates are usually higher in medium-income countries rather than in high-income countries, with the highest rates (>4 deaths/100 000 habitants per year) observed in Ukraine, Republic of Moldova, Romania, Cuba, Latvia, Russia, Estonia and Mexico for both males and females (Figure 8.3a,b) [4].

In Europe, data from the Automated Childhood Cancer Information System (ACCIS) Project

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
Published by Cambridge University Press. 

**Table 8.1** International Classification of Childhood Cancer, third edition

| Group | Subgroup | Description |
|---|---|---|
| I | – | Leukemias, myeloproliferative diseases and myelodisplastic diseases |
| | Ia | Lymphoid leukemias |
| | Ib | Acute myeloid leukemias |
| | Ic | Chronic myeloproliferative diseases |
| | Id | Myleodisplastic syndrome and other myeloproliferative diseases |
| | Ie | Unspecified and other specified leukemias |
| II | – | Lymphomas and reticuloendothelial neoplasms |
| | IIa | Hodgkin's lymphomas |
| | IIb | Non-Hodgkin's lymphomas (except Burkitt's lymphoma) |
| | IIc | Burkitt's lymphoma |
| | IId | Miscellaneous lymphoreticular neoplasms |
| | IIe | Unspecified lymphomas |
| III | – | Central nervous system and miscellaneous intracranial and intraspinal neoplasms |
| | IIIa | Ependymomas and choroid plexus tumor |
| | IIIb | Astrocytomas |
| | IIIc | Intracranial and intraspinal embryonal tumors |
| | IIId | Other gliomas |
| | IIIe | Other specified intracranial and intraspinal neoplasms |
| | IIIf | Unspecified intracranial and intraspinal neoplasms |
| IV | – | Neuroblastoma and other peripheral nervous cell tumors |
| | IVa | Neuroblastoma and ganglioneuroblastoma |
| | IVb | Other peripheral nervous cell tumors |
| V | – | Retinoblastoma |
| VI | – | Renal tumors |
| | VIa | Nephroblastoma and other nonepithelial renal tumors |
| | VIb | Renal carcinomas |
| | Vic | Unspecified malignant renal tumors |
| VII | – | Hepatic tumors |
| | VIIa | Hepatoblastoma |
| | VIIb | Hepatic carcinomas |
| | VIIc | Unspecified malignant hepatic tumors |
| IX | – | Soft tissue and other extraosseous sarcomas |
| | IXa | Rhabdomyosarcomas |
| | IXb | Fibrosarcomas, peripheral nerve sheath tumors and other fibrous neoplasms |
| | IXc | Kaposi's sarcoma |
| | IXd | Other specified soft tissue sarcomas |
| | IXe | Unspecified soft tissue sarcomas |
| X | – | Germ cell tumors, trophoblastic tumors and neoplasms of gonads |
| | Xa | Intracranial and intraspinal germ cell tumors |
| | Xb | Malignant extracranial and extragonadal germ cell tumors |
| | Xc | Malignant gonadal germ cell tumors |
| | Xd | Gonadal carcinomas |
| | Xe | Other and unspecified malignant gonadal tumors |
| XI | – | Other malignant epithelial neoplasms and malignant melanomas |
| | XIa | Adrenocortical carcinomas |
| | XIb | Thyroid carcinomas |
| | XIb | Nasopharyngeal carcinomas |
| | XIc | Malignant melanomas |
| | XId | Skin carcinomas |
| | XIe | Other and unspecified carcinomas |
| XII | – | Other and unspecified malignant neoplasms |
| | XIIa | Other specified malignant tumors |
| | XIIb | Other unspecified malignant tumors |

With permission from Steliarova-Foucher *et al.* [2].

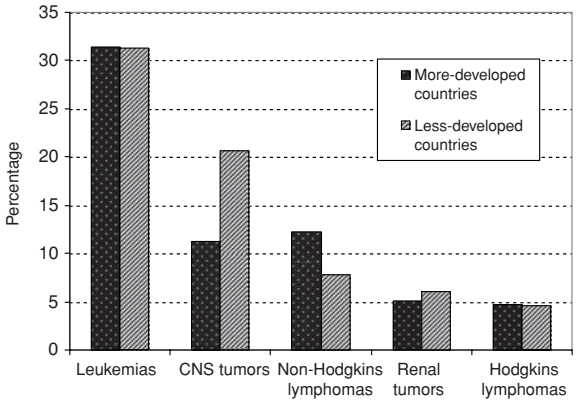


**Figure 8.1** Childhood cancer types distribution in more developed and less developed countries, 2002. CNS, central nervous system. With permission from Ferlay *et al.* [3].

show an increase in overall incidence of childhood cancer in all ages [5]. Mean age-standardized rates (per million) were 118 in the 1970s, 124 in the 1980s and 139 in the 1990s, corresponding to an annual increase of 1.0% during this period. Significant increases were observed for leukemias (average annual percentage change [AAPC] = 0.7%, $P < 0.001$), lymphomas (AAPC = 1.3%, $P < 0.001$), neuroblastomas (AAPC = 2.0%, $P < 0.001$), soft tissue sarcomas (AAPC = 1.8%, $P < 0.001$), germ cell tumors (AAPC = 2.3%, $P < 0.001$), renal tumors (AAPC = 1.1%, $P = 0.017$), hepatic tumors (AAPC = 1.0%, $P = 0.027$) and bone tumors (AAPC = 0.4%, $P = 0.023$). For CNS tumors increases were higher in Eastern Europe (AAPC = 2.5%, $P < 0.001$) compared to Western Europe

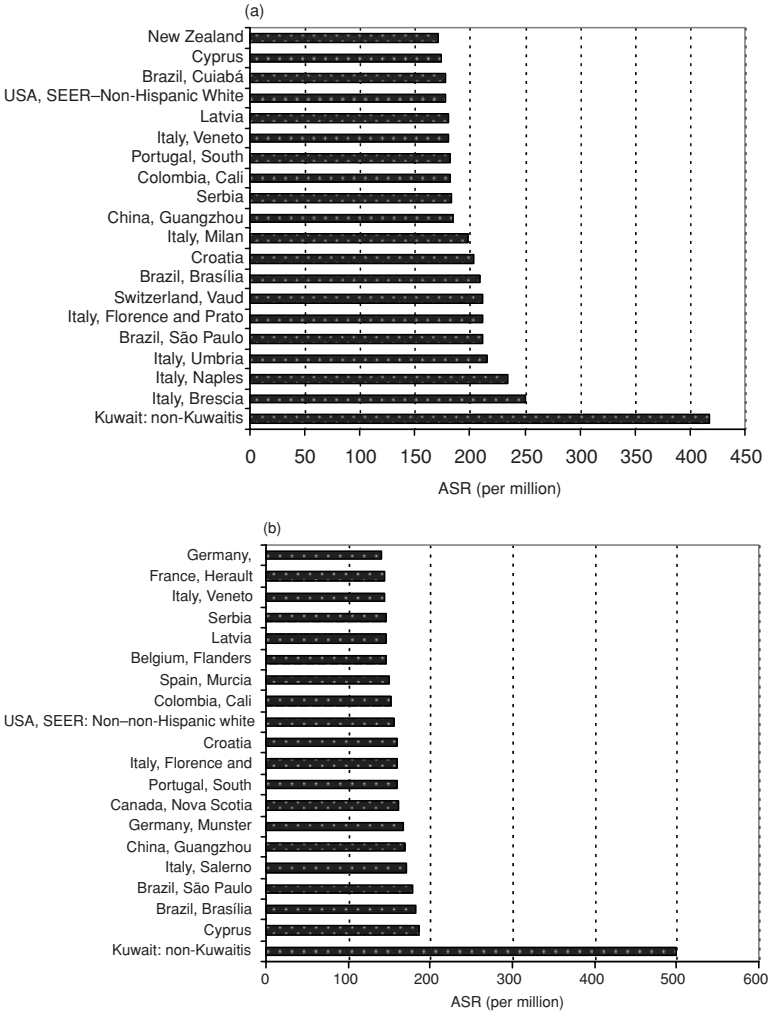


**Figure 8.2** Populations with the highest age-standardized incidence rate of childhood cancer (0–14 years), 1998–2002. (a) All sites but skin, males. (b) All sites but skin, females. ASR, age-standardized annual incidence rate. With permission from Curado *et al.* [1].

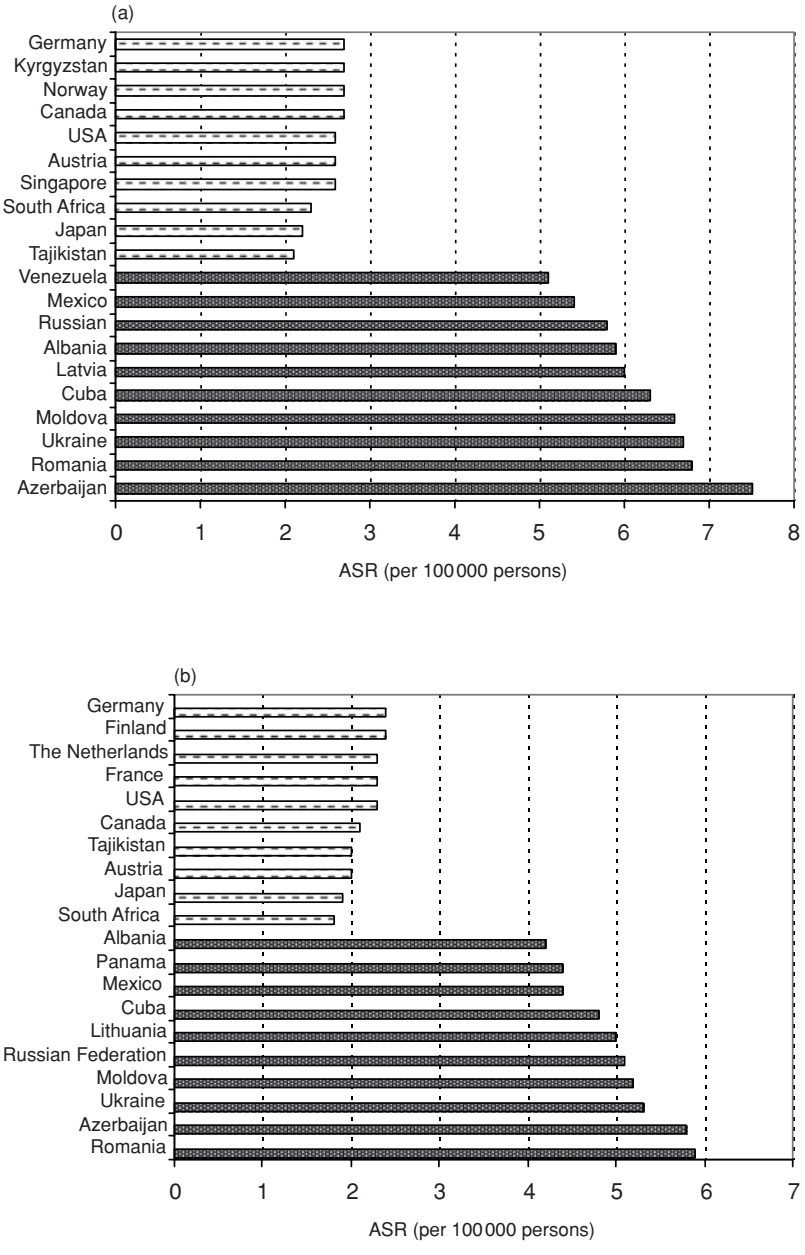


**Figure 8.3** Age-standardized cancer mortality rates in children (0–14 years) in selected populations, 2000–2004. (a) Males. (b) Females. ASR, age-standardized annual incidence rate. With permission from Ferlay [4].

(AAPC = 0.8%, $P < 0.001$), while for retinoblastoma increases were observed only for children aged <1 year (AAPC = 1.1%, $P = 0.018$) [5]. In the USA, from 1992 to 2004, no significant increase was observed for total childhood cancer incidence (<19 years of age) (AAPC = 0.4%; 95% CI, –0.1 to 0.8%). However, significant increases were observed for hepatoblastomas (AAPC = 4.3%, 95% CI 0.2–8.7%) and melanomas (AAPC = 2.8%, 95% CI 0.5–5.1%) [6].

## Cancer in adolescents and young adults

Incidence rates in the 15–24 year age group range from 10.7 (Poona, India) to 47.2 (Queensland, Australia) per 100 000 persons for males and between 9.9 (Harbin, China) and 60.4 (Iceland) per 100 000 persons for females [1]. The populations with the highest incidence rates for males and females are reported in Figure 8.4a and 8.4b, respectively. A

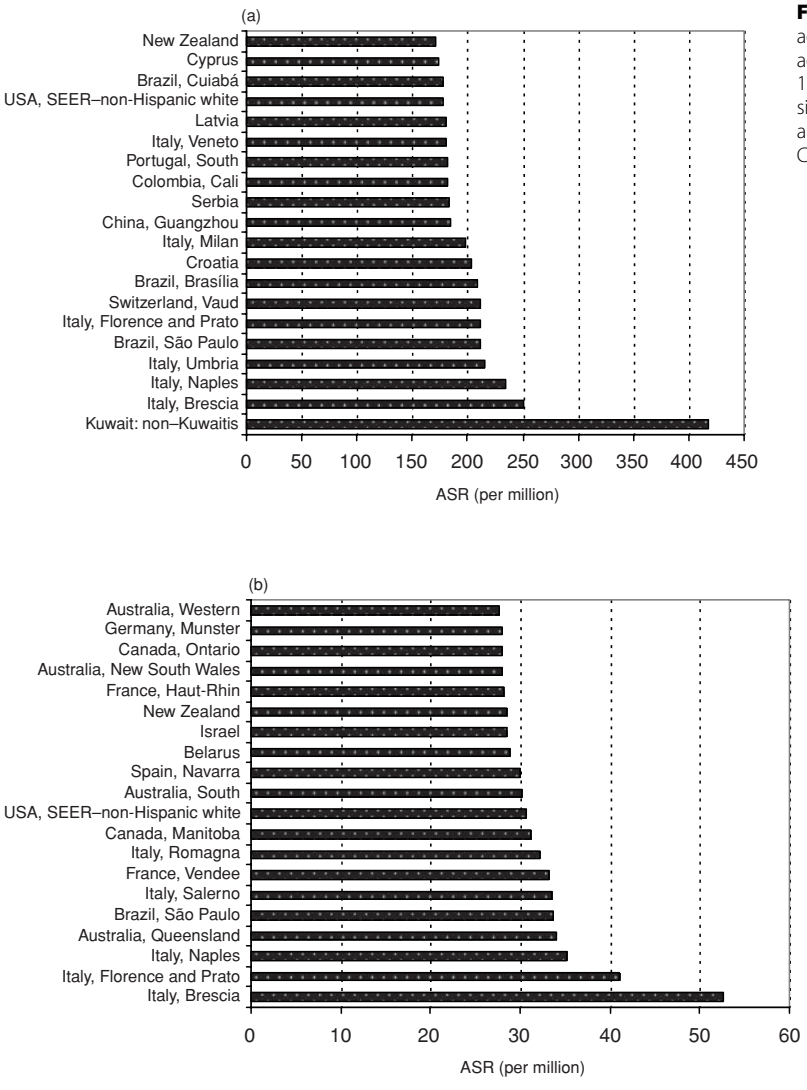


**Figure 8.4** Populations with the highest age-standardized incidence rate of cancer in adolescents and young adults (15–24 years), 1998–2002. (a) All sites but skin, males. (b) All sites but skin, females. ASR, age-standardized annual incidence rate. With permission from Curado *et al.* [1].

classification system for adolescents and young adults with cancer has recently been developed [7] and is summarized in Table 8.2. In a recent publication from the US Surveillance Epidemiology and End Results (SEER) program, analyzing all invasive cancers that occurred in the age group 15–29 between 1975 and 2000, the most frequent tumor types were lymphoma (20%), invasive skin cancer (15%), cancer of the male genital system (11%) and cancer of the endocrine glands (11%) [8]. As in children, high mortality rates are experienced in medium-income countries such as Ukraine, Costa Rica, Mexico, Russia, Romania, Lithuania, Ecuador, Venezuela and Cuba, with mortality rates >5.5 and 7.5/100 000 persons for males and females, respectively (Figure 8.5a,b) [4].

Steliarova-Foucher *et al.* described an increase in cancer incidence for adolescents (15–19 years) in Europe between 1970 and 1999, with remarkable increases for carcinomas (AAPC = 3.9%, $P < 0.001$), lymphomas (AAPC = 2.4%, $P < 0.001$) and germ cell tumors (AAPC = 3.9%, $P < 0.001$) [5]. Bleyer *et al.*, based on data from SEER for the period 1975–2001, has described an increase in incidence of cancer in adolescents and young adults in the USA for age groups 15–19 years, 20–24 years and 25–29 years [8]. Soft tissue sarcomas and lymphomas were responsible for most of the increase observed for males in the

**Table 8.2** International Classification for Cancers in Adolescents and Young Adults

| Group | Subgroup | Description |
|---|---|---|
| 1 | – | Leukemias |
| | 1.1 | Acute lymphoid leukemia |
| | 1.2 | Acute myeloid leukemia |
| | 1.3 | Other and unspecified leukemia |
| 2 | – | Lymphomas |
| | 2.1 | Non-Hodgkin's lymphoma |
| | 2.2 | Hodgkin's disease |
| 3 | – | Central nervous system and other intracranial and intraspinal neoplasms |
| | 3.1 | Astrocytoma |
| | 3.2 | Other gliomas |
| | 3.3 | Ependymoma |
| | 3.4 | Medulloblastoma and other primitive neuroectodermal tumors |
| | 3.5 | Other and unspecified malignant intracranial neoplasms |
| | 3.6 | Non-malignant intracranial and intraspinal neoplasms |
| 4 | – | Osseous and chondromatous neoplasms, Ewing tumors and other neoplasms of bone |
| | 4.1 | Osteosarcoma |
| | 4.2 | Chondrosarcoma |
| | 4.3 | Ewing's tumor |
| | 4.4 | Other specified and unspecified bone tumors |
| 5 | – | Soft tissue sarcoma |
| | 5.1 | Fibromatous neoplasms |
| | 5.2 | Rhabdomyosarcoma |
| | 5.3 | Other soft tissue sarcomas |
| 6 | – | Germ cell and trophoblastic neoplasms |
| | 6.1 | Gonadal germ cell and trophoblastic neoplasms |
| | 6.2 | Germ cell and trophoblastic neoplasms of non-gonadal sites |
| 7 | – | Melanoma and skin carcinoma |
| | 7.1 | Melanoma |
| | 7.2 | Skin carcinoma |
| 8 | – | Carcinomas (except of skin) |
| | 8.1 | Carcinoma of thyroid |
| | 8.2 | Other carcinoma of head and neck |
| | 8.3 | Carcinoma of trachea, bronchus, lung and pleura |
| | 8.4 | Carcinoma of breast |
| | 8.5 | Carcinoma of genito-urinary tract |
| | 8.6 | Carcinoma of gastrointestinal tract |
| | 8.7 | Carcinoma of other and ill-defined sites not elsewhere classified |
| 9 | – | Miscellaneous specified neoplasms |
| | 9.1 | Embryonal tumors not elsewhere classified |
| | 9.2 | Other rare miscellaneous specified neoplasms |
| 10 | – | Unspecified malignant neoplasms |

With permission from Birch *et al.* [7].

age group 25–29 years, suggesting that this finding can be attributed to the emergence of acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma and NHLs [8].

## Survival

Survival for children with cancer has continued to improve over the past 20 years. In high-income countries, since the late 1990s, 5-year survival rates are close to 80% [9].

In Europe, according to data from 83 population-based cancer registries in 23 countries participating in the EUROCARE-4 project, 5-year survival rates for all cancers combined was 81% for children and 87% for adolescents and young adults. Among children under 15 years of age, very high survival rates were observed for retinoblastoma (97.5%),

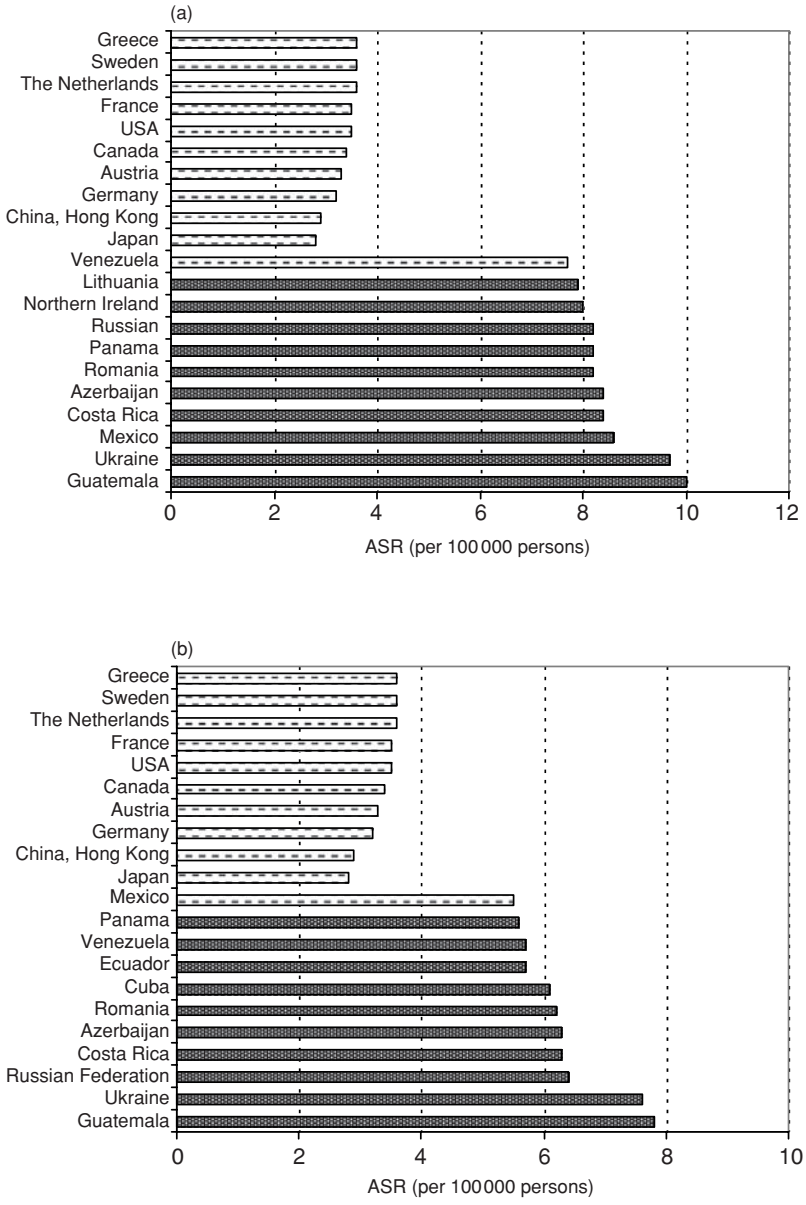


**Figure 8.5** Age-standardized cancer mortality rates in adolescents and young adults (15–24 years) in selected populations, 2000–2004. (a) Males. (b) Females. ASR, age-standardized annual incidence rate. With permission from Ferlay [4].

Hodgkin's lymphoma (95.2%), Wilms' tumor (89.1%) and lymphoid leukemia (85.4%), while less favorable figures were observed for CNS nervous system tumors (66.8%) and acute myeloid leukemias (66.8%) [10]. In general, survival rates are lower in Central and Eastern Europe than in Western Europe. In the USA, for children and adolescents (0–19 years) diagnosed 1999–2005, registered into 17 SEER Cancer Registries, the 5-year relative survival rate for all cancers was 79.3%, ranging from 59.6 (hepatic tumors) to 97.0% (retinoblastoma) [11]. Contrasting with the finding for adults, in which cancer survival for European adults is lower than that observed in USA, no systematic differences were observed between these two regions regarding childhood cancer survival [12].

Unfortunately, in spite of advances in diagnosis and treatment, survival rates are still poor in low and middle-income countries, such as India (Chennai, 40%) [13]. In several low-income

countries (Bangladesh, Philippines, Senegal, Tanzania and Vietnam), 5-year overall survival is as low as 10% [14].

# Risk factors: childhood cancer

## Ionizing radiation

### Intrauterine and postnatal exposure

Ionizing radiation is a known cause of cancer and other adverse effects. It is one of the most extensively studied human carcinogens and may account for about 3% of all cancers [15]. Epidemiological studies have shown an association between exposure to medical radiation during pregnancy and risk of childhood cancer in offspring. The first association between exposure of pregnant women to abdominal diagnostic X-rays and pediatric cancer mortality in offspring was reported in the Oxford Survey of Childhood Cancer (OSCC), a nationwide case-control study including all children (<16 years), residing in England, Scotland and Wales, who died from cancer during 1953–81 and matched living controls [16]. The causal nature of the association has been discussed, since the evidence in favor of an association derives almost exclusively from case-control studies, whereas practically all cohort studies failed to replicate it [17]. However, the most recent analysis of OSCC data confirmed that intrauterine X-ray examination is associated with an increase of 40% in the risk of childhood cancer [16]. Beginning at the 1970s, abdominal X-ray and pelvimetry were progressively replaced by obstetrical sonography and case-control studies carried out in UK [18], China [19], USA and Canada [20] did not show evidence of an increased risk of childhood cancer overall or leukemia associated to this exposure.

The relation between exposure to diagnostic radiation during early childhood and the subsequent risk of developing a pediatric cancer is less obvious [21]. Linet *et al.* have recently reviewed the relevant evidence, based on 22 studies [21]. Only one case-control study from China reported an increased risk of all childhood cancers [19]. Among the five studies addressing the risk of acute lymphoblastic leukemia (ALL), only one reported an association with exposure to two or more diagnostic X-rays [22]. Concerning brain tumors, an increased risk was observed in one study for children exposed to diagnostic skull X-rays at least 5 years before diagnosis (OR = 6.7, 95% CI 1.6–27.3), although reverse causality, i.e. the appearance of symptoms linked to the brain tumor led to a skull X-ray could not be ruled out [23].

### Preconception exposure

Linet *et al.* have called attention to the difficulties in assessing results from studies evaluating the association between maternal preconception exposure to diagnostic medical radiation and childhood cancer [21]. According to these authors, the data are scarce and difficult to interpret due to lack of standardization of the time window of exposure, lack of emphasis on key anatomic sites of exposure, absence of assessment of the medical indication for the radiological examination and failure to adjust the results for therapeutic and occupational radiation exposure [21]. The early results of the OSCC indicated an association between abdominal X-ray exposures prior to marriage and childhood cancer [24], but a subsequent analysis of data collected during the course of OSCC did not support this finding (OR of all childhood cancer for preconception X-ray 1.1, 95% CI 0.9–1.2) [25]. Most of the further studies also did not detect an association [19, 26–30].

## Extremely low-frequency electromagnetic fields

Extremely low-frequency magnetic fields (ELF-MF) has been classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans mainly based on epidemiological evidence derived from studies on childhood leukemia. The first study assessing the association between exposure to ELF-MF and childhood leukemia was published in 1979 [31], and results from more than 20 epidemiological studies focusing on this topic have been summarized in two distinct pooled analyses [32, 33]. Greenland *et al.* pooled original individual data from 12 studies, and summary estimates showed no association when comparing 0.1–0.2 and 0.2–0.3 microtesla (μT) categories with the 0.0–0.1 μT category, but a significant association was found for the comparison between >0.3 μT and 0.0–0.1 μT (OR = 1.7, 95% CI 1.2–2.3) [33]. In the same year, Ahlbom *et al.* reported results of an analysis including individual data from 9 studies published between 1993 and 1999, describing that for residential magnetic field levels <0.4 μT no evidence of an increased risk of childhood leukemia was found. On the other hand, residential exposures

≥0.4 μT a year before diagnosis, conferred a twofold higher risk of childhood leukemia [32].

In a recently published report, including data from a case-control study carried out in Germany, no increased risk of childhood cancer was associated with preconception parental occupational exposure to ELF-MF [34]. In addition, an exploratory meta-analysis performed by the same authors, including other four studies that evaluated the association between paternal occupational exposure to ELF-MF and childhood leukemia, resulted in a pooled risk estimate of 1.35 (95% CI 0.95–1.91) [34].

## Parental occupational exposure

Parental occupational exposure might be related to cancer in children. Several mechanisms can be cited on the explanation about how these exposures might lead to an increased risk of cancer in the offspring. These mechanisms comprises carcinogenic or mutagenic damage to either paternal or maternal germ cells before pregnancy, injury to the fetus through transplacental exposure during pregnancy and also direct exposure of the children through contamination of breast milk or parental clothes [35].

However, results from studies assessing these associations are somewhat non-homogeneous, mainly because of poor assessment of exposures, low frequency of exposure, failure to assess exposure windows and multiple comparisons [36].

Occupations and corresponding exposures are largely more studied for fathers than for mothers [36]. For childhood leukemias, positive associations with paternal exposure to diverse chemical agents have been found, including paints, solvents, pesticides and working in motor-vehicle related jobs [37], but the results are not consistent among studies.

Brain tumors have been linked to paternal exposure to paints and pesticides and employment in the petroleum and chemical industries [37]. Exposure to pesticides and organic chemicals during paternal hobbies has been associated with risk of brain tumor in children [38].

Paternal exposures to hydrocarbons have been associated with an increased risk of neuroblastoma in some reports [39, 40]. Spitz and Johnson described an increased risk of neuroblastoma associated with jobs linked to moderate exposures to aromatic and aliphatic hydrocarbons [40]. A large case-control study conducted by the Children's Cancer Group and the Pediatric Oncology Group has found significant increased risks of neuroblastoma for fathers working as landscapers and groundskeepers (OR = 2.3, 95% CI 1.0–5.2), as well as for mothers employed as hairdressers and barbers (OR = 2.8, 95% CI 1.2–6.3) [41]. In a recent literature review, including 47 articles that have evaluated risk factors for neuroblastoma, Heck *et al.* concluded that there is suggestive evidence of positive association with paternal exposure to volatile and non-volatile hydrocarbons, wood dusts and solders [42]. On the other hand, findings from recent studies carried out in the UK did not support the role of parental occupational exposures as important risk factor for neuroblastoma [43], Wilms' tumor [44] or retinoblastoma [45]. MacCarthy *et al.* assessed 32 paternal occupational exposure groups, among which only leather was associated with neuroblastoma (OR = 5.0, 95% CI 1.1–46.9) [43]. The same group of investigators has conducted another case-control study to assess the role of these exposures on the risk of retinoblastoma and have found an association only with paternal occupational exposure to oil mists in metal working (OR = 1.85, 95% CI 1.05–3.36) [45]. Furthermore, data from the National Registry of Childhood Tumors have served as a basis for a case-control study, including approximately 2500 pairs of cases and controls, in which no associations between paternal occupational exposures and Wilms' tumor was found [44]. In conclusion, epidemiological studies linking occupational parental exposure to childhood cancer suggest several potential links, but for none the evidence can be considered strong.

The association between pesticides and childhood cancer was first suggested after the publication of a few case reports in which leukemias and other childhood tumors were reported following the utilization of insecticides and herbicides in the home and yard [46–48]. An increased risk of childhood leukemia has been found for fathers occupationally exposed to pesticides prior to and during pregnancy [49], as well as for parental pesticide exposures at home or in gardens [49].

Other types of childhood cancer including neuroblastoma, Wilms' tumor, soft tissue sarcoma, Ewing's sarcoma, NHLs, CNS tumors, colorectal and testicular cancer have also been associated with exposure to pesticides, based on evidence from case reports and case-control studies. Although these studies are subject to several caveats, including non-specific pesticide exposure information, small numbers of exposed

subjects and potential for information bias, it is remarkable that many of the reported increased risks are of greater magnitude than those observed in studies with the adult population, suggesting that children may be particularly susceptible to the carcinogenic effects of pesticides [50].

A recent literature review about the association between pesticides and childhood cancers, including 36 studies published between 1998 and 2006, has concluded that definite conclusions cannot be drawn from the existing evidence [51]. According to this author, although several studies suggest associations between pesticides and childhood cancers, there is no consistency regarding tumor types and implied agents, and estimates derived from case-control studies are usually higher than those obtained in cohort studies, particularly concerning studies on childhood leukemia.

## Infections

It has been long proposed that infections might cause childhood leukemia and NHLs, based on two non-mutually exclusive mechanisms. Greaves hypothesized that a delayed exposure to common infections in infancy and early childhood leads to an increased risk of ALL [52], while Kinlen suggested that a large influx of people into relatively isolated areas (population mixing) might facilitate the spread of a viral infection that occurs when infected and susceptible individuals come in contact with each other, leading to an increased risk of childhood leukemia [53]. Although isolated studies have suggested varicella [54], influenza [55] and Epstein–Barr virus (EBV) [56] as possible etiological factors, no single agent has been compellingly implied as a cause.

Epstein–Barr virus is linked to Burkitt's lymphoma (BL), and this association is based on sero-epidemiological studies and the identification of EBV genomes in tumor specimens from individuals living in endemic areas [57]. Endemic BL is predominantly found among children living in equatorial Africa and in Papua New Guinea, where it is strongly associated with endemic malaria. In these areas, pediatric lymphomas account for up to 80% of all cancers in children. On the other hand, areas with low incidence of BL have a much lower (<30%) proportion of BL associated with EBV [58], while areas with an intermediate pattern, like South America, show percentages ranging between 25 and 70% [59–62].

Epstein–Barr virus has also been also identified as a causal agent for nasopharyngeal carcinoma (NPC). The etiological link between NPC and EBV was first proposed on the basis of serological evidence. High antibody titres of immunoglobulin G (IgG) and immunoglobulin A (IgA) against early antigen or viral capsid antigen are frequently observed in individuals with NPC, particularly those with the undifferentiated tumors. This association was later confirmed by the finding of EBV genomes in tumor cells [63].

Human immunodeficiency virus (HIV) infection has also been associated with the development of malignancies in children, although the occurrence is less frequent than in adults. Non-Hodgkin's lymphomas are the most frequent cancer in children with AIDS, followed by leiomyomas and leiomyosarcomas, which are also clearly associated with EBV infection [64]. Kaposi's sarcoma is rare in children [64] and its etiology probably involves infection with human herpesvirus 8. Therefore, it has been suggested that the risk for this cancer is higher among those HIV-positive children who were born to mothers in high-risk groups (heterosexual transmission via a bisexual partner) or who became infected postnatally through contaminated blood [65, 66].

## Lifestyle factors

The effects of maternal lifestyle during pregnancy on embryonic and fetal development are well known and, therefore, effects on the subsequent risk of cancer in the offspring might be expected [57]. Several features of maternal lifestyle during pregnancy have been studied regarding their association with childhood cancer, including diet, breastfeeding, smoking and alcohol consumption and the use of cosmetics. In addition, paternal exposures during the preconception period are also hypothesized as having effects on the risk of germ cell mutations [57], and habits like tobacco smoking and alcohol consumption have also been assessed regarding the risk of childhood cancer.

### Diet and breastfeeding

Parker conducted a non-systematic review of published studies on the association between leukemia/lymphoma and breastfeeding and suggested that 25% of the 500 annual cases of childhood acute leukemia or lymphoma registered at the UK could be prevented if prevalence of breastfeeding was increased to 100% [67]. Results of an analysis conducted within the

context of the UK Childhood Cancer Study, including 3500 cases (1637 with leukemia) and 6964 controls, have demonstrated a small protective effect for breastfed children, both for leukemia (OR = 0.89; 95% CI 0.80–1.00) and for all cancers combined (OR = 0.92; 95% CI 0.84–1.00) [68]. A first meta-analysis comprising 14 case-control studies (including 6835 ALL cases and 1216 acute myeloid leukemia [AML] cases) have shown a negative association between long-term breastfeeding (>6 months) and both ALL (OR = 0.76; 95% CI 0.68–0.84) and AML risk (OR = 0.85; 95% CI 0.73–0.98) [69]. In 2005, Martin *et al.* published another meta-analysis, including 26 publications (92% were case-control studies), suggesting that breastfeeding was associated with a 9% risk reduction for ALL (OR = 0.91; 95% CI 0.84–0.98), 24% for Hodgkin's disease (OR = 0.76; 95% CI 0.60–0.97) and 41% for neuroblastoma (OR = 0.59; 95% CI 0.44–0.78) [70].

Results from the Northern California Childhood Leukemia Study, including 282 case-control sets of children, in which maternal diet (12 months before pregnancy) was evaluated through a food-frequency questionnaire, have shown that the consumption of vegetables (OR = 0.65; 95% CI 0.50–0.84), fruits (OR = 0.81; 95% CI 0.65–1.00), proteins (OR = 0.55; 95% CI 0.32–0.96) and legumes (OR = 0.75; 95% CI 0.59–0.95) were associated with a reduced risk of ALL [71]. Another study from the same group have also investigated the role of child's diet on leukemia risk and authors have reported that the regular consumption of oranges/bananas (OR = 0.49; 95% CI 0.26–0.94) and orange juice (OR = 0.54; 95% CI 0.31–0.94) during the first 2 years of life were both associated with a reduction in risk of childhood leukemia [72]. In addition, a recent Taiwanese report has pointed out the role of the child's consumption of cured/smoked meat and fish on increasing the risk of acute childhood leukemia (OR = 1.74; 95% CI 1.15–2.64), while it also has shown a protective effect linked to a frequent consumption of vegetables (OR = 0.55; 95% CI 0.37–0.83) and bean-curd food (OR = 0.55; 95% CI 0.34–0.89) [73].

Several studies have investigated the role of maternal diet during pregnancy as a risk or protective factor in relation to pediatric CNS tumors, and the most compelling and studied hypothesis concerns the high risk of disease in the offspring of those mothers with a high intake of N-nitroso compounds and precursors. Results from an international collaborative case-control study have shown specific associations between cured meat consumption and pilocytic astrocytomas (OR = 2.5; 95% CI 1.1–5.8) or ependymomas (OR = 2.0, 95% CI 1.4–2.9), as well as negative associations between cruciferous vegetables and anaplastic astrocytomas (OR = 0.4; 95% CI 0.3–0.7) and also fresh fish consumption and malignant gliomas (OR = 0.5; 95% CI 0.3–0.6). Oil products intake was associated with an increased risk of medulloblastoma (OR = 1.5; 95% CI 1.0–2.2) [74].

### Parental tobacco smoking

Cigarette smoking has been shown to increase oxidative DNA damage in human sperm cells [75]. The link between paternal cigarette smoking and childhood cancer has not been sufficiently evaluated, and the results of the epidemiological studies have been contradictory. Most of the studies on maternal smoking and childhood leukemia did not find a significant positive association and some have shown a protective association. On the other hand, studies assessing paternal smoking and childhood leukemia reported positive associations more frequently [76].

In the UK, three studies that have analyzed data from OSCC did not find a statistically significant association between maternal smoking and childhood cancer [77–79]. However, significant positive associations were found for the paternal smoking habit, with all three studies showing increased risks for fathers who were moderate or heavy smokers [77–79]. A subsequent report from the Inter-Regional Epidemiological Study of Childhood Cancer (IRESCC) confirmed the findings for paternal smoking, showing a significant positive trend between the risk of childhood cancer and paternal daily consumption of cigarettes before the pregnancy [80].

A case-control study conducted by the UK Childhood Cancer Study (UKCCS) has found a statistically significant association between hepatoblastoma and maternal smoking (OR = 2.7) and also an increased and stronger risk when both parents had a smoking habit (OR = 4.7) [81]. This last finding was subsequently confirmed in a publication with data from the OSCC, in which an increased risk of hepatoblastoma when both parents were smokers was shown (OR = 2.7, 95% CI 1.2–6.1) [82].

There is no consistent evidence regarding a possible association between childhood brain tumors and maternal smoking [57]. However, Preston-Martin *et al.* have reported a positive association with mothers living in a household where someone else smoked

during the pregnancy of the index child [83]. Recently, the findings from the ESCALE study provide additional evidence for a role of paternal smoking during the year prior to birth in childhood CNS tumors (≥20 cigarettes/day, OR = 1.4, 95%CI 1.0–2.1) and more markedly for astrocytomas (≥20 cigarettes/day, OR = 3.2, 95% CI 1.2–9.1) and ependymomas (≥20 cigarettes/day, OR = 2.6, 95% CI 1.2–5.9) [84].

Concerning retinoblastoma, Bunin *et al.* did not find a significant association between maternal (OR = 1.1, 95% CI 0.6–2.1) or paternal smoking (OR = 1.2, 95% CI 0.7–2.3) during pregnancy [85]. However, a recently published record linkage study including the New South Wales Central Cancer Registry and the New South Wales Midwives Data Collection, in which more than one million babies, born between 1994 and 2005, were matched to 948 cancer cases, has shown that maternal smoking was significantly associated with retinoblastoma (OR = 2.2, 95% CI 1.2–4.1) [86].

### Parental alcohol consumption

The association between parental alcohol consumption and childhood cancer, in particular childhood leukemias, has been assessed in several studies, but the overall evidence is not conclusive. More recent studies, however, have shown an increased risk of childhood leukemia linked to maternal drinking during pregnancy. MacArthur *et al.* have reported that both preconceptional (OR = 1.37; 95% CI 0.99–1.90) and during pregnancy maternal drinking (OR = 1.39; 95% CI 1.01–1.93) were associated with an increased risk of childhood leukemia, with a dose-response effect observed for increasing weekly consumption [87]. A French population-based case-control study has found an increased risk of ALL in the offspring of mothers who reported a consumption of more than 1 drink/day (OR = 2.8; 95% CI 1.8–5.9) [88], while other studies have reported no association [89, 90]. Infante-Rivard *et al.* have additionally investigated the interaction between prenatal parental exposure and children's polymorphisms in the metabolizing genes *GSTM1* and *CYP2E1* [91]. Conversely to what was observed in the French study, the authors have suggested that alcohol consumption during pregnancy can have a protective effect, particularly for those mothers who drank wine (OR = 0.7, 95% CI 0.5–0.9). However, exposure was associated with an increased risk of disease for those mothers with *GSTM1* genotype (drinking any alcohol, second trimester, OR = 2.3, 95% CI 1.0–5.1; third trimester, OR = 2.4, 95% CI 1.1–5.4) [91].

Parental alcohol consumption has also been associated with other tumors like brain tumors, neuroblastomas, Wilms' tumors, germ cell tumors, soft tissue sarcomas, bone tumors, retinoblastomas and hepatoblastomas [92]. Overall, there was no strong evidence for a positive association. Ten of the thirty-three studies observed an increase in risk of childhood cancer associated to parental alcohol consumption. Seven studies reported associations with maternal consumption for the following tumors: leukemia (five studies), brain tumor (two studies) and neuroblastoma (two studies). Conversely, a protective effect was described in four studies. In addition, three studies reported a positive association with paternal alcohol consumption, whereas no study showed a protective effect for this exposure [92].

### Recreational drugs

Parental illicit drugs use has been associated with several types of childhood cancer, including leukemia, neuroblastoma, brain tumor and rhabdomyosarcoma. A case-control study conducted by Robison *et al.* reported that the maternal use of mind-altering drugs (mainly marijuana) prior to or during the index pregnancy was associated to an increased risk of acute non-lymphoblastic leukemia [93]. Kuijten *et al.*, using data from a case-control study conducted in the USA in the late 1980s, reported that maternal use of any recreational drug, and particularly marijuana, was associated with an increased risk of astrocytoma in the offspring (OR = 2.8, 95% CI 0.9–9.9) [94].

A case-control study carried out in the USA, including 322 cases and 322 matched controls, has shown that maternal use of marijuana during the year before the child birth conferred a threefold higher risk of rhabdomyosarcoma (RMS) (95% CI 1.4–6.5) [95]. Maternal use of cocaine was also associated with RMS (OR = 5.1, 95% CI 1.0–25.0). Both paternal marijuana smoking (OR = 2.0, 95% CI 1.3–3.3) and cocaine use (OR = 2.1, 95% CI 0.9–4.9) were also associated with an increased risk of this type of tumor [95].

More recently, findings form a study conducted in the USA showed that maternal use of marijuana in the first trimester may also increase the risk of their child developing neuroblastoma (OR = 4.8, 95% CI 1.6–16.5) [96].

Zahm and Devesa have reiterated the difficulties needed to make an accurate assessment of these

exposures and the need for additional studies [97]. On the other hand, these authors have emphasized the need of continuing prevention efforts even in the absence of consistent evidence of association with childhood cancer, due to the other health problems already definitely associated with the use of illicit drugs [97].

Overall, a role of lifestyle factors in the etiology of childhood cancer is plausible, and the evidence is stronger for paternal tobacco smoking and breastfeeding. For other aspects of lifestyle, notably diet, the available data do not allow a conclusion at present.

## Risk factors for the most frequent cancers in adolescents and young adults

Lymphoma, melanoma and testicular, cervical and thyroid cancers account for the vast majority of cancers in this age group (65% of the total). Among them, melanoma, cervical cancer and lymphoma are the tumor types more strongly linked to environmental and lifestyle agents (such as ultraviolet light [UV], human papilloma virus [HPV], HIV and EBV) [8]. Risk factors for lymphoma have been reviewed in the previous section.

### Melanoma

The etiology of melanomas is not fully understood, although epidemiological studies have pointed out several risk factors for developing the disease, including high solar exposure in early childhood, sunburns, light hair and eye color, pale white skin and sunbed use [98]. However, the major environmental risk factor for melanoma is undoubtedly the exposure to UV radiation [98]. A meta-analysis published in 2005, including 57 studies from Europe, North America, Australia, New Zealand, Argentina, Brazil and Israel, has found a significant increase in the risk of melanoma associated with total sun exposure (RR = 1.34, 95% CI 1.02–1.77), with studies published after 1990 showing a stronger effect (RR = 1.75, 95% CI 1.31–2.35). Intermittent sun exposure (RR = 1.61, 95% CI 1.31–1.99) and sunburn history (RR = 2.03, 95% CI 1.73–2.37) were also confirmed as significant risk factors for this cancer [99].

### Testicular cancer

Cryptorchidism is the best characterized risk factor for testicular cancer. This association was first documented at the beginning of the nineteenth century and the presence of this congenital abnormality confers an increase in the risk of testicular cancer of 2–11-fold. Risk is higher if cryptorchidism is bilateral or if the condition is not treated before the age of 11 years. Other lifestyle and environmental factors have been investigated but there is no evidence of a consistent increase in the risk of testicular cancer. In an extensive review, Garner *et al.* have summarized the epidemiological evidence, describing a possible etiological role for maternal characteristics and exposures (endogenous estrogen level prenatal smoking), characteristics of the child (early height and decreased levels of androgen), occupational exposures (pesticides) and reproductive factors (age at puberty, age at pregnancy, infertility) [100].

### Cervical cancer

Infection with HPV is the main risk factor for cervical cancer, and viral DNA is found in almost 100% of the tumors. Human papilloma virus oncogenic types 16, 18, 31, 33 and 35 are the most associated with invasive cervical carcinomas, with the types 16 and 18 causing approximately 70% of the cancers worldwide. Human papilloma virus is now recognized as the most common sexually transmitted infection in most populations. Infected women usually clear the infection within 2 years, but those who become chronically infected with high-risk HPV types are at greatest risk for developing cervical cancer [101]. Female adolescents are one of the population groups with highest risk of becoming infected by HPV. It is estimated that 75% of all new HPV infections are diagnosed among those women aged 15–24 years, with oncogenic HPV types accounting for nearly 50% of the infections among adolescents. Recently, two vaccines against HPV were developed: a quadrivalent (protecting against HPVs 6, 11, 16 and 18) and a bivalent vaccine (HPVs 16 and 18). Clinical trials have shown almost 100% of efficacy of the vaccine in preventing high-grade, pre-cancerous cervical lesions, caused by the HPV types prevented by the vaccination, for women naïve to HPV types included in the vaccine. Both vaccines are now licensed for use in women more than 90 countries. Since 2006, the quadrivalent vaccine is approved for use in the USA, in girls aged 9–26 years, and the Advisory Committee on Immunization Practices (ACIP) currently recommends that all 11–12 year-old girls receive this vaccine

routinely, with a "catch up" use in women aged 13–26 years [102].

## Thyroid cancer

The only established risk factor for thyroid cancer, particular for papillary carcinomas, is ionizing radiation. Numerous reports have described the epidemiological aspects of thyroid cancer in areas associated with nuclear plants, accidents and atomic bombs. Studies that have assessed thyroid cancer incidence after the explosion of atomic bombs in Hiroshima and Nagasaki have demonstrated that the risk was highest among individual aged <10 years at the time of exposure. Moreover, a striking increase in thyroid cancer incidence among children was registered in areas surrounding Chernobyl, after the power plant accident in 1986. In Belarus, the incidence rate previous to the accident was 1 new case/million per year and it has increased to more than 100 new cases/million per year in the post-accident period. Diagnostic and therapeutic uses of radiation are also associated with an increased risk of thyroid cancer, even if low doses are applied [103].

# Conclusions

Cancer is the second commonest cause of death, after accidents, among children in high-income countries, while in low and medium-income countries, improvements in the control of infectious diseases and perinatal mortality will lead to the emergence of cancer in children as an important public health problem [57]. Incidence rates of childhood cancer have been increasing in the last decades in most countries. It is unclear whether this reflects a real phenomenon or an artifact due to better diagnosis and reporting, with improvement of the quality of cancer registries [5]. Current knowledge on etiological factors remains limited, particularly for tumors other than leukemia and CNS neoplasms. Known causes of childhood cancer include ionizing radiation, several infectious agents and possibly some behavioral factors; however, they explain only a small proportion of the cases. Future collaborative studies should be conducted, aiming to have enough power to test specific hypothesis as well as to integrate knowledge from different areas, including pediatric oncologists, epidemiologists and investigators working on basic research. Etiological hypotheses would be better tested in prospective studies, but very large populations of newborn and children need to be followed up to study a sufficiently large number of cases. Recently, investigators involved in prospective studies of children have formed the International Childhood Cancer Cohort Consortium (IC4), to promote large-scale collaboration between existing cohort studies [104]. Incorporation of biomarkers of exposure and early effect and investigation of interactions between environmental exposure and genetic predisposition factors represent additional avenues for future studies on the etiology of childhood cancer.

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Chapter 9

# Life and death in the germ line

## Apoptosis and the origins of DNA damage in human spermatozoa

R. J. Aitken and B. J. Curry

## Introduction: apoptosis and DNA damage in human spermatozoa

Apoptosis, a physiological process for the controlled deletion of cells, is critical for the regulation of cell numbers, the management of morphogenesis during embryonic development and the orchestration of many cellular processes in the adult. Spermatogenesis, the production of functional spermatozoa from spermatogonial stem cells is no exception. It appears that a functional apoptotic pathway is necessary for normal spermatogenesis to develop and without it infertility ensues. Apoptosis also plays a crucial role in the maintenance of the testis and its response to external toxicants as well as in the programmed senescence of terminally differentiated spermatozoa. This chapter will focus specifically on how apoptosis affects sperm quality and function, and the implications of this process for both embryonic development and the health and well-being of the offspring.

A great deal of data has accumulated in recent years suggesting that human spermatozoa can exhibit some of the hallmarks of apoptosis including activation of caspases 1, 3, 8 and 9 [1, 2], annexin-V binding [3, 4], mitochondrial generation of reactive oxygen species (ROS) [5] and DNA fragmentation [6–8]. The latter is potentially extremely important because DNA damage in the male germ line has been associated with a wide variety of adverse biological and clinical outcomes. Thus DNA damage in human spermatozoa has been correlated with poor fertilization and impaired embryonic development to the blastocyst stage [9, 10] as well as with the incidence of subsequent miscarriage [11–13]. Furthermore, even if such pregnancies do carry to term, the presence of DNA damage in the spermatozoa at the moment of conception has been linked with developmental abnormalities in the offspring leading to a wide range of different pathologies, including childhood cancer [12].

## DNA damage in spermatozoa, assisted reproductive technology and embryonic development

This apparent association between DNA damage in spermatozoa and the health and well-being of any progeny is particularly significant in the context of assisted reproductive technology (ART), which has come to dominate the therapeutic landscape for infertile couples. Thus, the developed nations of the world are currently seeing an exponential increase in the use of ART to treat human infertility to such an extent that 1 in 80 children born in the USA, 1 in 50 born in Sweden, 1 in 40 born in Australia and 1 in 24 born in Denmark are the product of this form of treatment. In 2003, more than 100 000 in vitro fertilization (IVF) cycles were reported from 399 clinics in the USA, resulting in the birth of more than 48 000 babies [14, 15]. Worldwide, this figure has now exceeded 200 000 births per annum and is continuing to rise. This massive uptake of ART may herald a developing public health crisis for at least two reasons. Firstly, human infertility is a complex multifactorial condition that is strongly impacted by genetic factors that ART will ensure are passed onto the progeny [16]. As a consequence, it is a biological certainty that the more we use assisted conception to treat one generation, the more

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we shall need it in the next. Secondly, in at least half of the patients referred for ART, there is a problem with the male partner. Since male subfertility is frequently associated with high levels of DNA damage in spermatozoa, the use of ART invariably means that DNA-damaged cells will be used to achieve conceptions in vitro that could never have occurred in vivo. This raises genuine concerns over the safety of IVF procedures should the DNA damage brought into the zygote by the fertilizing spermatozoon subsequently lead to disordered embryonic growth and development [15].

We already know that the incidence of birth defects following assisted conception is double that seen in the naturally conceived population [17], and there is good evidence for an increase in imprinting disorders, notably the Beckwith–Wiedemann and Angelman's syndromes, in such children [18, 19]. Infants produced by ART are also significantly more likely to be admitted to a neonatal intensive care unit, to be hospitalized and to stay in hospital longer than their naturally conceived counterparts [20]. Large studies of Scandinavian populations using record linkage have also shown an increase in the hospitalization of ART offspring in infancy and early childhood compared with spontaneously conceived children [21–23]. Such increases in morbidity cannot be completely explained by multiple births because singletons are similarly affected [23]. Furthermore, recent independent investigations have shown an eightfold increase in the incidence of undescended testicles in boys conceived by intracytoplasmic sperm injection (ICSI) [24], while another study has demonstrated abnormal retinal vascularization in such children [25]. Notwithstanding the concerns raised in these publications, it must also be stressed that other authors have been more reassuring about the normality of ICSI children, particularly in terms of their motor skills and cognitive development [26–28]. Such a mixed picture for the safety of ART arises from the literature for a number of reasons:

1. The incidence of definable events such as overt birth defects, hospitalizations or significant pathologies such as cancer is low.
2. The study populations are generally small.
3. The number of confounding variables influencing the health and well-being of children is great.
4. The duration of follow-up is short.
5. The instruments used for assessing defects in the embryo (incidence of miscarriage, birth defects, overt changes in the health and behavior of the offspring) are insensitive.

If we assume that the genetic/epigenetic damage associated with ART is randomly distributed throughout the genome then the chances that a specific gene will be directly affected is low, given that ~95% of the genome is non-coding. It is even less likely that if a mutation does occur in a gene, it will be dominant and cause a detectable phenotypic or behavioral change in the F1 generation. Clinically, the incidence of even the more common spontaneous dominant mutations, such as achondroplasia, approximates to 1 in every 10 000–30 000 births in the population at large [29]. Thus, if phenotypic change is the criterion, then most ART studies are simply not adequately powered to determine whether this form of therapy has a significant effect on the normality of the offspring. In this situation, the absence of evidence cannot be taken as evidence of the absence of problems with ART, and we should proceed on the assumption that this form of therapy carries significant risks that have to be quantified and addressed. The notion that DNA damage in human spermatozoa constitutes part of this risk is supported by clinical evidence generated around two major paradigms: aging and smoking.

## Age, DNA damage and disease in the progeny

As men age, they do not stop producing spermatozoa. However, the quality of the gametes they do produce deteriorates, leading to a loss of fertility [30] and increased levels of DNA damage in the spermatozoa [31]. The latter has been consistently observed to correlate with age across a number of studies, using a variety of different methods to detect the DNA strand breaks. Thus Singh *et al.* employed a Comet assay to demonstrate a significant correlation between age and DNA damage in human spermatozoa [31]. In the same vein, Schmid *et al.* found older men to have increased alkali-labile sites or single-strand DNA breaks in their spermatozoa using the same assay [32]. Others have employed the sperm chromatin structure assay (SCSA) to demonstrate a similar relationship between DNA damage and male age [33]. Furthermore, both human and rodent data have revealed age-related increases in the number of spermatozoa with chromosomal breaks and fragments [34]. A detailed analysis of chromosome 1 also found that the frequency of sperm

carrying breaks, segmental duplications and deletions was significantly higher among older men compared with their younger counterparts [35]. In particular, the frequency of spermatozoa carrying breaks within the 1q12 fragile-site region nearly doubled in older men. In contrast to female gametes, there was no effect of age on the frequency of sperm with numerical chromosomal abnormalities [35].

This abundant evidence for an effect of paternal age on DNA damage in the germ line is important because a father's age appears to be a major determinant of the health and well-being of his offspring. Indeed, for many decades we have been aware that paternal age has a dramatic influence on the incidence of spontaneous dominant genetic mutations such as achondroplasia, Apert's syndrome (acrocephalosyndactyly) and multiple endocrine neoplasia. For example, an analysis of 154 consecutive cases of dominant genetic mutations to determine the parent of origin, revealed that in every single case the mutation could be traced back to the male germ line, never the female. Moreover, the appearance of these mutations was found to be an exponential function of the age of the father. Such mutations appear to be created by replication error in the spermatogonial stem cell population, followed by clonal expansion of mutant germ cells as they enter a "selfish" pathway of proliferation [36, 37]. Of course, the genetic damage occurring in human spermatozoa as a function of paternal age is not only associated with dominant genetic mutations. Age is also associated with an increase in the incidence of complex polygenic neurological conditions in the offspring including epilepsy, spontaneous schizophrenia, bipolar disease and autism [12, 38–41] as well as an increased rate of death in the F1 generation associated with congenital malformations, injury and poisoning [42].

It is clear from the foregoing, that paternal age is associated with high rates of DNA damage in spermatozoa and that such age-dependent genetic damage to the male gamete is, in turn, associated with a wide range of clinical pathologies in the offspring, from dominant genetic mutations to complex neurological disorders. This age-dependent DNA damage is thought to be caused by oxidative stress originating from a combination of diminished antioxidant protection and elevated levels of ROS generation by the spermatozoa [43, 44]. Oxidative stress also appears to be at the heart of another factor known to increase DNA damage in the male germ line, smoking.

### Smoking, DNA damage and disease in the progeny

Heavy paternal smoking is thought to place the body under systemic oxidative attack causing a significant depletion of antioxidant vitamins such as vitamin C and E [45–47]. One of the consequences of the resultant stress is thought to be DNA fragmentation and formation of the oxidized base adduct 8-hydroxy-2'-deoxyguanosine (8OHdG) in the spermatozoa [46]. In addition, benzo[a]pyrenes derived from cigarette smoke can form adducts with sperm DNA, once metabolically activated by the cytochrome P450 system [48]. The clinical significance of these sperm DNA adducts can be found in the positive correlations that have been repeatedly observed between paternal smoking and serious morbidity in the offspring, including childhood cancer [49–53].

## Is DNA damage related to apoptosis in the germ line?

In summary, the above clinical data provide circumstantial evidence that DNA fragmentation in human spermatozoa is correlated with adverse clinical outcomes ranging from miscarriage to cancer. These conclusions are also supported by animal data indicating that the experimental induction of oxidative DNA damage in spermatozoa with cigarette smoke, alcohol or the experimental deletion of antioxidant enzymes, such as glutathione peroxidase 5, can subsequently lead to disruptions of embryonic growth resulting in high rates of abortion and the appearance of birth defects [54–56]. Given that these associations between sperm DNA damage and abnormalities of embryonic development clearly exist, it is now critical that we gain an understanding of how this DNA damage is generated in human spermatozoa and, in the context of this review, the role of apoptosis in the prosecution of this process.

### Nature of the DNA damage

In order to understand the origins of DNA damage in the male germ line we first have to understand its precise nature. A small number of studies have actually examined the damaged DNA from human spermatozoa in the search for clues as to its origins. These studies have revealed that the major base adduct present in human spermatozoa is 8OHdG, a marker of oxidative

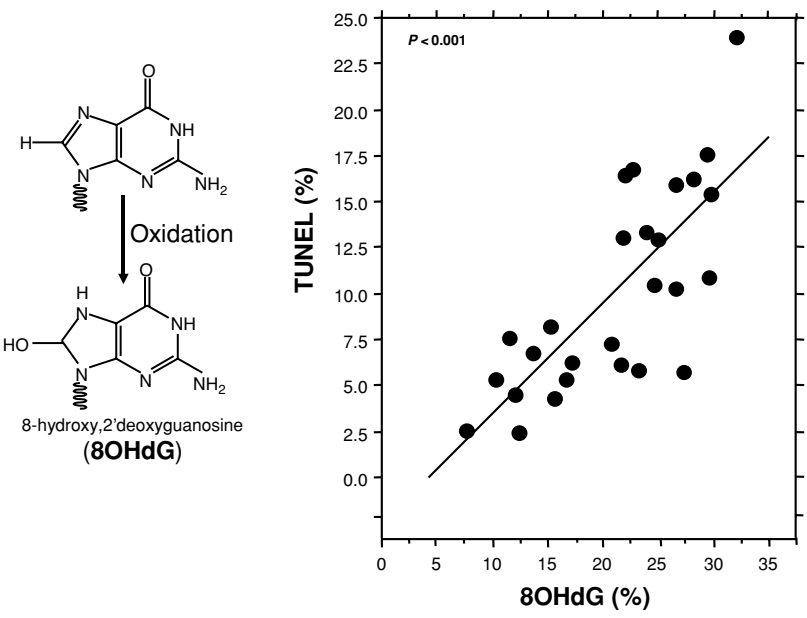


**Figure 9.1** The powerful correlation observed by De Iuliis *et al.* [57] between DNA fragmentation in human spermatozoa and oxidative DNA base damage as indicated by expression of 8-hydroxy-2′-deoxyguanosine (8OHdG).

stress. The levels of 8OHdG expression in human spermatozoa are consistently found to be elevated in the spermatozoa of infertile men [57, 58]. Moreover, the presence of this oxidized base adduct has been found to exhibit an extremely strong correlation with both DNA damage as measured by the TUNEL assay and chromatin protamination as assessed by chromomycin (CMA3) staining [57]. Indeed, the correlation between TUNEL and 8OHdG formation is so strong that we have been forced to conclude that a majority of DNA damage in the male germ line is oxidatively induced (Figure 9.1 [57]).

Besides 8OHdG, biochemical analyses of DNA from infertility patients have also revealed the presence of two ethenonucleosides: 1,N6-ethenoadenosine and 1,N2-ethenoguanosine. These compounds probably arise from a reaction with 4-hydroxy-2-nonenal, the main aldehyde released during lipid peroxidation [59]. These findings are again consistent with oxidative stress being a major factor in the etiology of DNA damage in the male germ line.

In another study, uncharacterized bulky DNA adducts were found to be significantly more common in the spermatozoa of male factor infertility patients than in a cohort of healthy donors [60]. Furthermore, a significant negative correlation was observed between these bulky DNA adducts and sperm concentration and sperm motility, among patients with impaired fertility [60]. In a further study, polycyclic aromatic hydrocarbon–DNA adducts were found to be more prevalent in infertile versus fertile men [61]. While the origin of some of the DNA adducts detected in human spermatozoa are clear, as in the case of smoking [48], we clearly still have a lot to learn about the causes of DNA damage in the germ line. The one thing that does appear to be certain is that a significant proportion of the spontaneous DNA damage seen in male infertility patients is oxidative in nature.

The link between DNA oxidation (8OHdG formation) and DNA strand breakage could be interpreted in one of two ways. First, the link may be causative. That is, the formation of oxidative base adducts disrupts DNA integrity by labilizing the glycosyl bond that attaches the base to the ribose unit with the resultant generation of an abasic site. Abasic sites have a strong destabilizing effect on the DNA backbone which can subsequently result in strand breaks. Alternatively the relationship may be indirect. Oxidative base damage and DNA fragmentation may simply be independent witnesses to the same fundamental underlying process – the ability of spermatozoa to undergo apoptosis. Under these circumstances the DNA strand breakage could be linked to endonucleases activated during an apoptotic cascade that was triggered by oxidative stress. Such considerations have encouraged us to consider the process of apoptosis in the male germ line as

these cells differentiate from spermatogonial stem cells to fully formed spermatozoa.

## Ability of the germ line to undergo apoptosis

The ability of germ cells to undergo apoptosis is expressed very early in life when the testes are differentiating and adjustments have to be made to achieve the optimal ratio of germ cells to Sertoli cells. During this developmental process, excess pre-meiotic spermatogonia are removed by an early wave of apoptosis that accompanies the first round of spermatogenesis in the testis. Functional deletion of the pro-apoptotic protein, Bax, or over-expression of anti-apoptotic factors such as BclxL or Bcl2, generates a male infertility phenotype associated with a perturbed germ cell to Sertoli cell ratio [62, 63]. This phenotype shows disordered seminiferous tubules filled with spermatogonia, but no mature haploid sperm, while other tubules lack any germ cells whatsoever. Later in life, p53 and Fas are involved in the removal of germ cells that are damaged as a result of exposure to environmental toxicants or chemotherapeutic agents [64]. A role for apoptosis in the etiology of spontaneous male infertility has also been suggested by virtue of the excessively high numbers of apoptotic germ cells observed in the testes of some infertile males [65]. It has also been suggested that the DNA damage that features so prominently in human spermatozoa is the result of an abortive apoptotic process that was initiated during spermatogenesis but failed to run to completion because the extensive remodeling of germ cells to produce spermatozoa, removes the intracellular machinery needed to effect cell death [66].

# Significance of spermiogenesis in the etiology of DNA damage

Spermiogenesis, the process by which round spermatids differentiate into spermatozoa, is a key event in the etiology of DNA damage in the male germ line. As indicated above, it is possible that some of the DNA breaks seen in spermatozoa are the result of an abortive apoptotic process initiated during spermiogenesis in response to some disruptive event. Equally, DNA fragmentation in spermatozoa may also be the result of unresolved strand breaks created during the normal process of spermiogenesis in order to relieve the torsional stresses involved in packaging a very large amount of DNA into a very small sperm head. Normally, these "physiological" strand breaks are corrected by a complex process involving H2Ax expression, formation of poly(ADP-ribose) by nuclear poly(ADP-ribose) polymerases and topoisomerase [67]. However, if spermiogenesis should be disrupted for some reason then the restoration of these cleavage sites might be impaired and the spermatozoa, lacking any capacity for DNA repair in their own right, will be released from the germinal epithelium still carrying the unresolved strand breaks.

Experimentally, if physiological strand break repair during spermiogenesis is impaired then spermatozoa are generated that exhibit high levels of DNA fragmentation. For example, it is known that the chromatin remodeling steps associated with spermiogenesis trigger poly(ADP-ribose) (PAR) formation, as an early event in DNA repair. Knockout mice deficient in enzymes involved in PAR metabolism (PARP1 [poly(ADP-ribose) polymerase]; PARG [poly(ADP-ribose) glycohydrolase, 110-kDa isoform]; or both display DNA strand breaks associated with varying degrees of subfertility [67]. Similarly the "transition" proteins that move into the sperm nucleus during spermiogenesis, between the removal of histones and the entry of protamines, are thought to play a key role in maintaining DNA integrity. If these proteins are functionally deleted then spermatozoa are generated with poor fertilizing potential, poor chromatin compaction and high levels of DNA fragmentation [68]. Such studies clearly indicate that the functional disruption of chromatin repair mechanisms operative during spermiogenesis can result in the genesis of spermatozoa carrying high levels of DNA fragmentation. However, even though disruption of DNA repair mechanisms during spermiogenesis can result in DNA-damaged spermatozoa, this does not necessarily mean that such mechanisms are actually operative in a clinical context.

Using the fluorescent probe CMA3 to monitor the efficiency of sperm protamination, many independent laboratories have generated data on the excellent correlations observed between DNA fragmentation in the one hand and poor chromatin remodeling during spermiogenesis on the other (Figure 9.2a–c). This proposed link between defective spermiogenesis and DNA damage is further supported by the fact that several independent studies have recorded

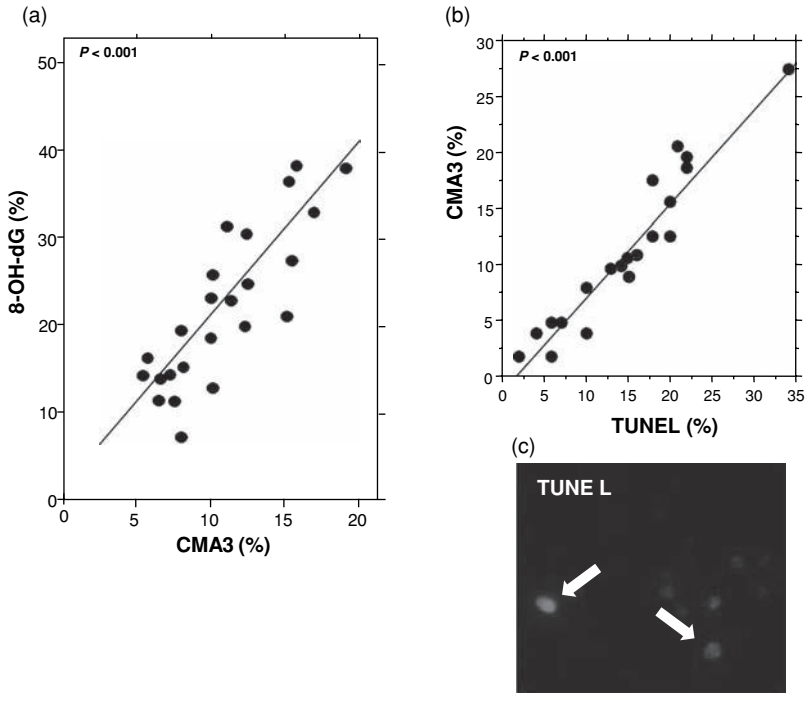


**Figure 9.2** The close relationship between the efficiency of chromatin remodeling, as monitored by chromomycin (CMA3) fluorescence and DNA damage in spermatozoa: (a) correlation between CMA3 and 8-hydroxy-2′-deoxyguanosine (8OHdG); (b) correlation between CMA3 and DNA fragmentation as measured by the TUNEL assay; (c) image of the TUNEL signals generated by human spermatozoa possessing damaged DNA (arrowed).

correlations between DNA damage in human spermatozoa and elements of the conventional semen profile (specifically sperm count and morphology) that, in turn, reflect the efficiency of the spermatogenic process.

Not only is the disruption of spermiogenesis correlated with DNA damage; it is specifically correlated with oxidative DNA damage as reflected by 8OHdG formation (Figure 9.2). We postulate that this relationship exists because the poorly remodeled chromatin detected by CMA3 is particularly vulnerable to oxidative attack by ROS originating from a number of potential sources including infiltrating leukocytes, depleted antioxidant systems and excessive free radical generation by the spermatozoa's own mitochondria. We further propose that of all these potential sources, the sperm mitochondria are the most important [5]. Experimental conditions associated with the induction of high levels of oxidative DNA damage, such as exposure to radio frequency electromagnetic radiation (RFEMR) [9] or the triggering of apoptosis through the suppression of PI3 kinase with wortmannin [R. J. Aitken, unpublished observations] invariably involve the release of considerable amounts of ROS from the sperm mitochondria.

## Importance of chromatin remodeling and oxidative stress

In light of the above considerations, DNA damage in human spermatozoa appears to have its origins in the testes and is associated with oxidative stress. We might interpret these data in two different ways:

1. *Impaired chromatin compaction and oxidative DNA damage are independent events*. It is possible that a variety of different factors are primarily responsible for impairing the quality of spermiogenesis (for example endocrine disruption, environmental toxicants, exposure to electromagnetic radiation or genetic mutations). The result of this disrupted spermiogenetic process is the production of spermatozoa with poorly remodeled chromatin that, because of the lack of DNA compaction, are particularly vulnerable to oxidative stress and the induction of 8OHdG formation. Under these circumstances the oxidative stress could come from the variety of sources mentioned above, including impaired antioxidant defenses or the enhanced generation of free radicals by either the spermatozoa

themselves or leukocytes in the immediate vicinity of these cells.

2. *Impaired chromatin compaction and oxidative DNA damage have a common origin in oxidative stress*. We have already cited evidence that DNA damage in spermatozoa is largely oxidatively induced. Whether spermiogenesis can also be adversely affected by oxidative stress is still a matter of debate. In male toxicology models involving, for example, the administration of streptozotocin [69], bacterial lipopolysaccharide [70], deltamethrin [71], methyl-parathion [72], aroclor 1254 [73], cyclophosphamide [74] or formaldehyde [75], oxidative stress is associated with the disruption of spermatogenesis via mechanisms that can be reversed by the administration of antioxidants such as lipoic acid [76], quercetin [69], Satureja khuzestanica essential oil [74] and melatonin [75]. In some of these experiments, the administration of an antioxidant both improved testicular function and reduced DNA damage in spermatozoa [74], suggesting that oxidative stress can underpin both the disruption of spermatogenesis and the induction of DNA damage in spermatozoa. In addition, the induction of oxidative stress with methyl parathion has been shown to specifically affect chromatin remodeling during spermiogenesis and induce DNA damage in spermatozoa [77]. Such results encourage speculation that oxidative stress is a major determinant of the efficacy of spermiogenesis which, when it becomes disrupted, results in the generation of spermatozoa that are vulnerable to oxidative stress, 8OHdG formation and, ultimately, DNA fragmentation (Figure 9.3 [78–80]). A powerful demonstration of the validity of this hypothesis has been provided by Zubkova and Robaire [81]. These authors induced systemic oxidative stress through the administration of the glutathione-depleting drug l-buthionine-[S,R]-sulphoximine (BSO). In response to the oxidative stress so generated, the rats exhibited evidence of both impaired spermiogenesis, as evidenced by an increase in CMA3 staining of the sperm chromatin, and increased DNA fragmentation.

Thus, oxidative stress can impair spermiogenesis and this, in turn, precipitates DNA fragmentation via a number of potential mechanisms (Figure 9.3):

1. Physiological DNA strand breaks introduced to facilitate DNA folding during spermiogenesis are left unresolved and persist in the mature gamete.
2. The defective spermatozoa generated as a result of disrupted spermiogenesis possess poorly protaminated chromatin that is vulnerable to free radical attack generating 8OHdG adducts, abasic sites and, ultimately, DNA fragmentation.
3. The defective spermatozoa, generated as a result of disrupted spermiogenesis, respond to oxidative stress by entering a default apoptotic pathway that results in endonuclease-mediated DNA cleavage.

## Importance of apoptosis in spermatozoa

There is no doubt that spermatozoa can exhibit many of the features of apoptosis and there is good evidence that oxidative stress can trigger apoptosis in these cells. Thus, exposure of human spermatozoa to hydrogen peroxide ($H_2O_2$) can readily trigger an apoptotic cascade characterized by the activation of caspase 3 and the appearance of annexin-V binding positivity [82]. We have also demonstrated that the activation of an apoptotic cascade following $H_2O_2$ exposure results in the induction of mitochondrial free radical generation [A. Koppers and R. J. Aitken, unpublished observations]. The activation of mitochondrial ROS generation, in turn, induces the formation of 8OHdG adducts in human sperm chromatin. We have observed this cascade of cause-and-effect involving oxidative stress, the activation of mitochondrial ROS generation and the induction of oxidized base adduct formation following: (1) the direct addition of oxidant ($H_2O_2$) to spermatozoa; (2) the indirect creation of oxidative stress with RFEMR [83]; and (3) the activation of apoptosis using the PI3 kinase inhibitor, wortmannin [A. Koppers and R. J. Aitken, unpublished observations]. We have also pursued this apoptotic cascade to determine whether the activation of caspases, mitochondrial ROS generation and phosphatidylserine (PS) externalization is followed by the activation of endonucleases that then move into the sperm nucleus to induce DNA fragmentation.

Surprisingly, this analysis revealed that apoptosis in spermatozoa differs in one important respect from this process in somatic cells, in that even though endonucleases may be released from the mitochondria

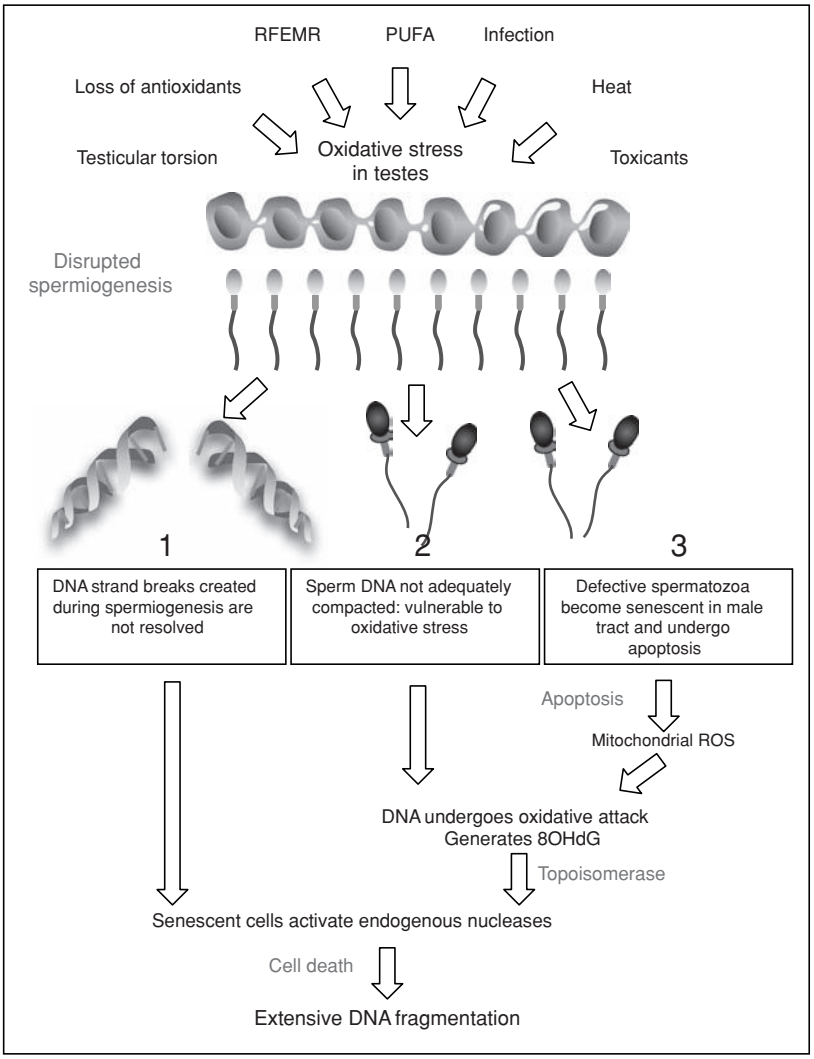


**Figure 9.3** Hypothesis for the creation of DNA damage in human spermatozoa. This hypothesis posits that a variety of different clinical, genetic and environmental factors can induce oxidative stress in the testis. One of the processes affected by oxidative stress is the differentiation of spermatozoa during spermiogenesis. As a result of disrupted spermiogenesis DNA damage will occur in the spermatozoa via at least three potential routes: (1) "Physiological", topoisomerase-mediated strand breaks that occur during spermiogenesis to relieve the torsional stresses associated with DNA packaging are not resolved and persist in the mature gamete. (2) Defective spermiogenesis generates spermatozoa possessing poorly compacted, inadequately protaminated DNA that is vulnerable to free radical attack originating from a variety of sources including depleted antioxidant protection, leukocytic infiltration, redox-cycling xenobiotics and ROS generation by the spermatozoa themselves. (3) Defective human spermatozoa respond to their imperfect state by prematurely engaging in a process of programmed senescence equivalent to apoptosis. During apoptosis caspases are activated, the spermatozoa lose their motility, phosphatidylserine (PS) externalization occurs and the mitochondria start to generate ROS. As a consequence of routes (2) and (3) the DNA is oxidatively attacked, generating the base adduct, 8-hydroxy-2′-deoxyguanosine (8OHdG), which ultimately leads to the creation of abasic sites and DNA strand breakage. In addition, we propose that as cells enter the terminal stages of senescence, topoisomerase, possibly acting in concert with endogenous endonucleases [78–80], completes the final destruction of the DNA.

(endonuclease G, apoptosis inducing factor) or activated in the cytosol (caspase-activated DNase) during the apoptotic cascade, the physical architecture of these cells prevents these nucleases from translocating to the sperm nucleus for two reasons:

1. The chromatin is so densely packed that proteins cannot penetrate into its internal structure.
2. The mitochondria and a majority of the cytoplasm are located in the sperm midpiece while the chromatin is located in the sperm head.

Thus, these enzymatic mediators of apoptotic DNA cleavage cannot be directly involved in the cleavage of sperm DNA. Rather, apoptosis is associated with mitochondrial ROS and 8OHdG formation which secondarily induces the non-enzymatic fragmentation of DNA following the creation of abasic sites. The only other possibility is that, in order to compensate for the inability of endonucleases to move into the sperm chromatin during apoptosis, spermatozoa are constructed with an endonuclease already integrated into the body of the chromatin, which only has to be activated for DNA cleavage to commence. In this context Sotolongo *et al.* and Shaman *et al.* have described endogenous nucleases that would fit these criteria [78, 79]. These authors envisage that this nuclease activity is initiated by topoisomerase IIB which becomes activated by divalent cations such as calcium or manganese [80]. The result of this topoisomerase activity is to cut the inter-toroid regions of sperm DNA to yield ~50 kb looped fragments. An uncharacterized second nuclease activity is then activated that further degrades the DNA in a process known as sperm DNA degradation (SDD). It is possible that these endonuclease activities are involved in the final stages of apoptosis when cell viability is being lost and the plasma membrane is no longer in a condition to exclude divalent cations such as calcium from the cells' interior (Figure 9.3). This observation would be in keeping with our recent observation that a vast majority of TUNEL positive cells in human sperm suspensions are, in fact, dead [84].

## Lives in the balance: apoptosis and pro-survival factors

In light of the foregoing discussion, we hypothesize that oxidative stress impairs the progress of spermiogenesis resulting in the generation of spermatozoa that may not only carry unresolved "physiological" DNA strand breaks acquired during early spermiogenesis, but also possess poorly compacted chromatin that is vulnerable to free radical attack. Such an attack could come from many quarters including infiltrating leukocytes, a lack of antioxidant protection or simply very prolonged periods of storage, i.e abstinence. It is also possible that spermatozoa created as a result of impaired spermiogenesis are more likely to enter an intrinsic apoptotic pathway in response to stress (Figure 9.3).

As far as we are aware, there are no specific chemical triggers for apoptosis in human spermatozoa. Rather entry into this pathway appears be triggered by cell senescence, which normally occurs in the female, not the male, reproductive tract. Following insemination, the female tract responds to the presence of millions of dead or moribund spermatozoa by triggering a massive leukocyte infiltration into the lower reproductive tract *post coitum* [85]. The phagocytic activity exhibited by these cells must be silent; in other words, the spermatozoa must be efficiently phagocytozed and removed from the tract in the absence of an oxidative burst or the production of pro-inflammatory cytokines. There are many examples of silent phagocytosis in biology, and a common feature of this phenomenon is the expression of apoptotic markers, such as PS, on the surface of the phagocytozed cell. This apoptotic marker is thought to instruct the phagocyte that the target cell should be engulfed in a non-phlogistic manner [86]. We therefore propose that the activation of this apoptotic cascade in senescent cells is an adaptation that permits the efficient removal of spermatozoa from the female tract by phagocytic leukocytes without provoking an inflammatory response. Viewed in this context, the DNA damage we see in the spermatozoa of male infertility patients with defective spermiogenesis could represent their premature entry into a pathway of programmed senescence involving activation of the intrinsic apoptotic cascade that should have occurred in the female tract. This hypothesis may explain why male infertility is so frequently accompanied by subclinical levels of leukocyte infiltration [87].

If entry into this truncated intrinsic apoptotic cascade is the default pathway for spermatozoa, we might ask what normally prevents them from becoming senescent. The answer to this question is that for the kind of prolonged survival that these cells exhibit in vivo, where they may have to spend up to a week in the epididymis waiting for ejaculation, followed by another week in the female tract waiting for an egg to arrive, exposure to pro-survival factors is critical. The latter are thought to maintain cell viability by driving a signal transduction pathway, which has PI3 kinase and Akt at its head. As long as these enzymes are phosphorylated they can, in turn, phosphorylate a key constituent of the apoptotic cascade called BAD. Phosphorylated BAD is bound to a regulatory protein called 14-3-3 and in this state cannot interact with the cell's mitochondria. However, if the cell should

become senescent for any reason then a reduction in PI3 kinase/Akt phosphorylation occurs which leads to the dephosphorylation of BAD. The latter then escapes from the embrace of its 14-3-3 regulator and moves rapidly to the mitochondria where it triggers pore formation, cytochrome C release and the initiation of apoptosis. If this is the case, then what are the pro-survival factors that normally keep spermatozoa from entering this default apoptotic pathway? The study of such factors is still in its infancy but one candidate to emerge from recent studies is prolactin. Spermatozoa possess several variants of the prolactin receptor (including their own unique isoform) and respond to the presence of this hormone with the stimulation of PI3 kinase/Akt phosphorylation and the prolongation of sperm survival [88].

## Conclusions

DNA damage in human spermatozoa has been correlated with a range of adverse clinical outcomes including subfertility, miscarriage and morbidity in the offspring. A majority of this DNA damage is oxidative and is correlated with the disruption of spermiogenesis and the generation of spermatozoa with poorly compacted chromatin. We hypothesize that the disruption of spermiogenesis is the result of oxidative stress, which can be induced by a wide range of factors including age, exposure to electromagnetic radiation in the form of heat or RFEMR, smoking, alcohol, insecticides, herbicides and heavy metals (Figure 9.3). The poorly protaminated spermatozoa generated as a consequence of defective spermiogenesis may not only carry unresolved "physiological" strand breaks but are also vulnerable to oxidative attack. We further hypothesize that this attack originates largely from the generation of ROS by defective spermatozoa as they prematurely enter a default pathway of programmed senescence characterized by a truncated apoptotic cascade featuring ROS generation, PS externalization and caspase activation. However, the endonucleases that translocate to the nucleus during somatic cell apoptosis are prevented from doing so in spermatozoa by virtue of the unique architecture of these cells. As a result, apoptosis in spermatozoa initially generates oxidative DNA damage followed by non-enzymatic DNA fragmentation. Enzymatic DNA cleavage may subsequently be induced by topoisomerase and other uncharacterized nucleases that are bound up in the structure of sperm chromatin. We propose that the activation of these enzymes occurs close to cell death or even post mortem in order to ensure the complete destruction of the cell (Figure 9.3). These concepts are not only novel but highly relevant to the safety of ART and the diagnosis, treatment and prevention of male factor infertility.

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Chapter 10

# Molecular aspects of follicular development

Zhongwei Huang and Dagan Wells

## Introduction

The follicle has a fundamental reproductive role in the ovary. It consists of the oocyte (in various stages of growth and development) and its surrounding layers of supportive cells, the granulosa cells (GCs), cumulus cells (CCs) (during the antral phase of follicular development) and thecal cells (TCs). These somatic cells perform essential roles in ensuring optimal development and maturation of the oocyte. They receive signals from the external environment and pass them on to the oocyte to orchestrate its growth and development until ovulation.

It is now widely recognized that there is bi-directional communication between the oocyte and surrounding somatic cells and that this is essential for the creation of a favorable follicular microenvironment in which the gamete can develop. Many complex and intricate molecular pathways are likely to be involved in the production of an optimal follicle and a viable gamete. This chapter aims to provide an insight into the molecular mechanisms likely to be involved in the various stages of follicular development, as determined from animal and human studies.

## The follicle develops in stages

During fetal life, the human ovary is populated by $\sim 7 \times 10^6$ oogonia as early as the 4th month of pregnancy with the highest mitotic activity seen just before meiosis commences [1]. These oogonia, present during the early second trimester, ultimately give rise to approximately 1 million primordial follicles at birth. The number of follicles continues to decline during childhood reaching ~300 000 at menarche [2].

Meiosis is initiated in the human fetal ovary at 11–12 weeks of gestation [3] when the oocytes enter prophase and go through synapsis with the exchange of genetic material between paired homologues. On completion of recombination, the oocyte progresses to diplotene of prophase I and enters a protracted arrest stage known as dictyate. At this stage such oocytes are known as primary oocytes and are surrounded by a single layer of flattened pre-granulosa cells. These primary oocytes and their associated cells make up the pool of primordial follicles, which a woman will utilize during the course of her reproductive life span. Primordial follicles remain quiescent for years until they are recruited to undergo further development during sexual maturation under the influence of pituitary gonadotrophin, follicle stimulating hormone (FSH) and luteinizing hormone (LH).

The pre-granulosa cells will continue to undergo cyto-differentiation and proliferation to support the oocyte during its early growth through the transition from the primordial to pre-antral follicular phase. During this transition, the morphology of the GCs changes from squamous to cuboidal type, as observed in the murine ovary [4]. The regulation of GC cyto-differentiation requires the actions of a number of hormones and growth factors [5] (discussed below).

Contact between GCs and the oocyte in its early growth is maintained via cytoplasmic processes penetrating the zona pellucida and forming gap junctions at the oocyte surface [6]. The gap junctions also form in increasing numbers between adjacent GCs to form an extensive network of inter-cellular connections. Through this network, substrates of low molecular weight such as amino acids and nucleotides are passed to the growing oocyte for its own synthesis of macromolecules as well as ribosomal and messenger RNA. The nutritional support and trafficking of macromolecules that this system allows may be particularly important for oocytes due to the avascular nature of the granulosa layer [7].

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As the follicle undergoes pre-antral to antral transition, the GCs continue to proliferate and as they do so, follicular fluid accumulates and coalesces to form a single follicular antrum. The follicular antrum enlarges during the antral phase but the oocyte does not increase in size during this period and remains suspended in fluid surrounded by the CCs (which differentiates from GCs during antrum formation). The CCs remain connected to the rim of GCs by a thin "stalk" of cells [7].

Follicle growth takes 85 days in humans and most follicles become atretic at some stage [8] with the oocyte continuing to play a critical role in follicular control and the regulation of oogenesis, ovulation rate and fecundity [9–11]. Only a minority of the developing follicles reach ovulation and, in most cases, only one will complete growth each month, reaching about 100 μm in diameter just before ovulation [12]. This oocyte will be ovulated in response to the mid-cycle LH surge, which effects the meiotic and cytoplasmic maturation of the oocyte. The oocyte will complete meiosis I and arrest at metaphase of meiosis II (MII) [13, 14]. Meiosis II is only resumed when fertilization occurs. The rest of the follicle collapses after ovulation and now becomes the corpus luteum. Both GCs and TCs contribute to the formation of the corpus luteum with a switch of morphology and endocrine functions.

The CCs continue to be associated with the oocyte after ovulation, assisting in oocyte transport along the Fallopian tube. The cumulus matrix and CCs also participate in fertilization by influencing spermatozoa binding and penetration of the cumulus oocyte complex (COC) [15, 16]. Subsequently, the intimate contact between the CCs and the oocyte is broken by the withdrawal of the cytoplasmic processes.

## From the formation of primordial follicles to the pre-antral phase of follicular development

The first primordial follicle in human foetuses is formed at 15–22 week gestation, at which point oocytes are enclosed by a single layer of pre-granulosa cells [17]. This only happens when primordial germ cells become oocytes upon the initiation of meiosis. Pre-granulosa cells are then recruited to the primary oocyte to form primordial follicles [18].

The formation of primordial follicles is controlled by retinoic acid (RA) [19]. Retinoic acid acts by binding to nuclear RA receptors which hetero-dimerize with nuclear retinoid X receptors (RXRα, β and γ). The heterodimer will bind to RA-response elements (RAREs) and thereby control the expression of RA-responsive genes [20]. Primordial follicle assembly (i.e. formation of primordial follicle) is inhibited by both progesterone and estrogen [21] but primordial follicle development is hormone independent [5].

The dynamics of the primordial follicle pool are not completely understood but involve the interplay between atresia, activation or maintenance of the primordial follicle by repressive signals like phosphatase and tensin homolog (PTEN), Foxo3 and anti-Müllerian hormone. Transition of primordial follicles to primary follicles requires activating signals which include stem cell factor (SCF) (also known as Kit ligand), basic fibroblast growth factor (bFGF) and bone morphogenetic proteins 4 and 7 (BMP-4 and -7) [22]. Primordial follicle activation involves changes to both GCs and the oocyte. Granulosa cells are stimulated to resume mitosis and assume a cuboidal shape, while the oocyte enlarges and cytoplasmic organelles start to proliferate and differentiate [23]. Subsequent recruitment and proliferation of TCs also take place [5] during primordial follicle activation.

Stem cell factor (Kit ligand) has been found to induce the primordial to primary follicle transition [24]. It appears to be a critical factor in stimulating follicular progression as GCs produce SCF, which appears to act on the oocyte, causing it to enlarge and initiate development [21, 24]. Furthermore, SCF also stimulates growth of TCs, their production of androgens and their expression of keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF). Keratinocyte growth factor, produced by the TCs, is able to stimulate the primordial to primary follicle transition [25] and acts on adjacent GCs as well. Furthermore, SCF expression is, in turn, stimulated by gonadotrophins, KGF and HGF [5], forming a positive feedback loop. Stem cell factor exerts its effects through the phosphatidyl-inositol-3-kinase (PI3K) pathway via phosphorylation of Akt, Foxo3a, glycogen synthase kinase 3α (GSK-3α) and GSK-3β [26]. The rapid oocyte growth during follicular activation is in accordance with the growth enhancing functions of the PI3K pathway [27, 28].

Platelet derived growth factor (PDGF) has been reported to activate the PI3K pathway [29] as well. The proteins and mRNA transcripts for two PDGF receptors are detected in human oocytes and the presence

of these receptors in human GCs suggests that PDGF might be involved in the activation of primordial follicles [30].

Another factor which, like SCF, appears to be a primordial follicle inducing factor is bFGF. Treatment with bFGF dramatically increases the number of primary follicles and decreases primordial follicle numbers [24, 31]. It is localized in the oocytes of primordial and primary follicles [31], acting on GCs, TCs and stroma. Basic fibroblast growth factor effects GCs' mitosis [32, 33], steroidogenesis [34], differentiation [35] and apoptosis [36].

Apoptosis and cellular proliferation of follicular cells are also influenced by leukemia inhibitory factor (LIF). This acts via a specific transducing receptor, GP130, feeding into the JAK–STAT pathway [37] in GCs and the oocyte [5]. Leukemia inhibitory factor promotes development of primordial follicles, a capacity which is enhanced by insulin [38].

A member of the transforming growth factor β (TGFβ) superfamily, bone morphogenetic protein 7 (BMP-7), also appears to have a role in early follicle development and involves a stroma cell interaction with the primordial follicle [39, 40]. Another member of the same family, BMP-4, is found to significantly increase primordial to primary follicle transition and is a required survival factor for oocytes [25]. However, anti-Müllerian hormone (AMH), also a member of the TGFβ superfamily appears to have the capacity to block primordial development. In addition, AMH is produced in the early secondary follicles, the pre-antral follicles and antral follicles [41] but is not expressed in the primordial follicle.

Tumor suppressors, tuberous sclerosis complexes (TSC) 1 and 2 are observed to suppress the activation of primordial follicles by suppressing mTOR (mammalian serine/threonine kinase mammalian target of rapamycin) complex 1 [42]. The TSC1–TSC2 complex suppresses the activity of mTOR complex 1 through a GTPase activating protein domain located in TSC2. The function of TSC1 is to stabilize TSC2 and protect it from ubiquitination and degradation [43].

Foxo3a, a downstream transcription target of the phosphatise and tensin homolog (PTEN)/PI3K pathway and a substrate of Akt, functions in oocytes to suppress follicular activation [44, 45]. Deletion of PTEN from oocytes of primordial follicles results in the premature activation of the entire primordial follicle pool by overgrowth of immature oocytes. The accelerated oocyte growth is found to be mediated by the enhanced activation of Akt and hence elevated phosphorylation (activation) of ribosomal protein S6 (rpS6), which is dependent on mTOR complex 1 [45]. Therefore, PTEN, which inhibits the actions of PI3K, governs follicular activation through control in the initiation of oocyte growth via PI3K, Akt and Foxo3a [46].

FOXL2, a single exon gene of 2.7 kb, belongs to the family of winged-helix/fork-head transcription factors [47] and is expressed in somatic cells of the developing human ovary before and during follicle formation [48]. This gene product is essential in pre-granulosa cells to mediate the squamous-to-cuboidal transition and proliferation. In the absence of FOXL2, the whole pool of primordial follicle gets prematurely activated without synchronizing with the differentiation and division of surrounding pre-granulosa cells. This results in follicles that lack multiple layers of functional GCs, ultimately leading to atresia of these follicles [49, 50]. It is likely that FOXL2 in pre-granulosa cells provides an inhibitory mechanism via gap junction or paracrine secretion and maintains the primordial follicles in a dormant state [42].

Upon activation of primordial follicles, progression from the primary (single GC layer) to secondary (pre-antral) stage of follicular development requires further oocyte expansion, GC proliferation and investment of an LH-responsive theca cell layer. These vital steps are not FSH dependent but remain critically dependent on paracrine signaling by members of the TGFβ superfamily [51]. In primary to pre-antral follicles, activin subunit (INHBA and INHBB) and AMH [41] gene expressions predominate [52].

Activins (homodimers or heterodimers of INHBA and INHBB) enhance GCs proliferation and promote their responsiveness to FSH. At the same instance, LH responsive thecal androgen synthesis will be suppressed. Appropriate stimulation by FSH will then divert the formation of activins to that of inhibins which promote androgen synthesis. Androgen synthesis is in turn linked to FSH augmentation of inhibin synthesis with the primary aim to ensure estrogen synthesis from the aromatizing of androgens [51].

Anti-Müllerian hormone expression peaks in GCs of secondary, pre-antral and small antral follicles <4 mm in diameter. In larger antral follicles (4–8 mm), AMH expression gradually disappears [53]. Anti-Müllerian hormone has been shown to suppress early stages of follicular growth and onset of responsiveness to FSH in vitro, exerting a controlling influence on the rate at which follicles become available

for pre-ovulatory development. The inhibitory effects of AMH on FSH-sensitivity of follicles may play this important role in the process of selection. A group of follicles with lower expression of AMH and hence, more sensitized to FSH, may be selected from the batch of AMH-producing growing follicles to continue growth up to the pre-ovulatory stage [41]. It appears that AMH produced by growing follicles may act as a negative paracrine feedback signal on neighboring primordial follicles to inhibit their recruitment [26].

Recent studies have shown that Smad2 and Smad3 are important mediators of TGFβ signaling in ovarian cells [54, 55]. They are expressed in GCs of pre-antral and small antral follicles but their expression becomes weak in the GCs of large antral follicles [54]. Anti-Müllerian hormone is likely to utilize isoforms of different Smads to mediate its effects on target cells [41]. Anti-Müllerian hormone decreases primordial to primary follicle transition by reducing the expression of stimulatory factors like SCF, bFGF and KGF [56].

A multitude of players are likely to be involved in this crucial stage in ovarian follicular development and once a primordial follicle has been recruited, it will be destined for further development. The oocyte commences its growth and secretes glycoproteins which condense around it to form the zona pellucida. This separates the oocyte from the rapidly proliferating and differentiating GCs during the pre-antral phase of follicular development.

The oocyte is shown to be a central regulator of follicular cell function, secreting soluble growth factors, oocyte secreted factors (OSF), which act on neighboring follicular cells to regulate a broad range of GC and CC functions, including differentiation, proliferation, apoptosis and luteinization [57]. This has been affirmed by two landmark studies which demonstrated that absence of two OSFs, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), causes sterility [58, 59]. Both GDF-9 and BMP-15 are from the TGFβ superfamily, are expressed in an oocyte-specific manner from a very early stage and play key roles in promoting follicle growth beyond the primary stage [60].

As mentioned before, bi-directional communication between the oocyte and surrounding somatic cells is maintained by cytoplasmic processes and gap junctions which facilitate the transfer of amino acids, nucleotides, glucose and metabolites essential for oocyte and follicle growth and maturation [61]. This communication is so crucial that genetic deletion of the oocyte specific gap junctional subunit, connexin-37, leads to female sterility in mice, resulting from a lack of mature follicles, failure to ovulate and development of numerous inappropriate corpora lutea. In addition, oocyte development in connexin-37 deficient mice is arrested before meiotic competence is achieved [62].

In addition to oocyte growth and GCs proliferation, the pre-antral follicle further increases its size and complexity through formation of a basal lamina between the GC and TC layers in addition to multiplication and differentiation of TCs into two theca layers: the inner vascular theca interna and the fibrous capsule, theca externa (Figure 10.1).

## Pre-antral to antral phase transition

The pre-antral follicle, also known as the secondary follicle (consisting of the oocyte surrounded by several layers of GCs and theca) continues its development and becomes increasingly FSH responsive. Further growth and continued survival are increasingly gonadotrophin dependent [63]. Activins in concert with other growth factors, such as insulin-like growth factors, augment multiple actions of FSH [64] on immature GCs including upregulation of FSH receptors and increased formation of inhibin-α subunit. Increasing inhibin-α diverts GCs from activin to inhibin biosynthesis and creates the potential for thecal androgen production to be stimulated by inhibin [52, 65]. The androgen produced is able to synergize with FSH in promoting GC mitosis and carbohydrate metabolism, including the increased formation of lactate required for energy production by the maturing oocyte [66].

The oocyte continues to accumulate large amounts of transcripts during its growth, while transcriptional activity ceases at the time of meiotic resumption [67]. It does not increase in size over the antral period but continues to actively synthesize RNA and turnover protein.

The GCs continue to proliferate resulting in a further increase in the size of the follicle. The GC and TC layers become gonadotrophin sensitized with the expression of FSH receptors in GCs and LH receptors in TCs, allowing these cells to assume steroidogenic roles. Follicular fluid, which is composed partly of GC secretions (including muco-polysaccharides) and partly serum transudate, starts to form as the GCs

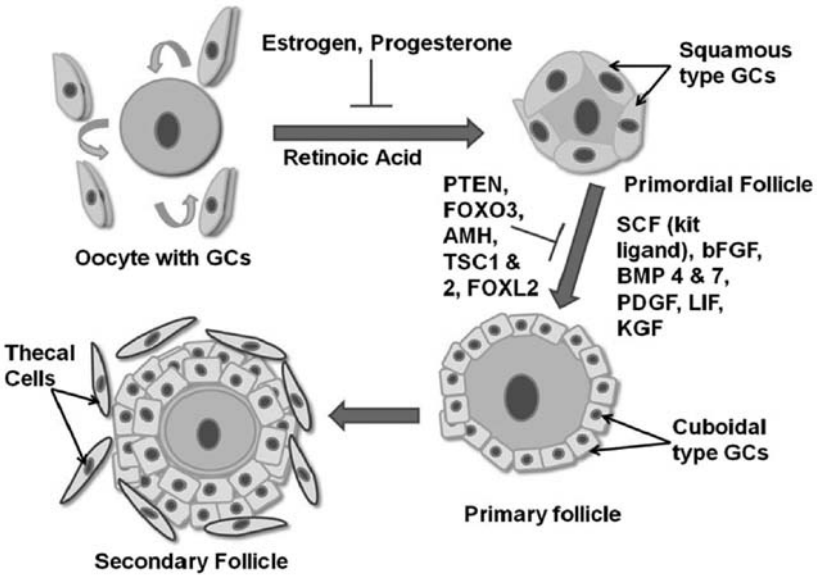


**Figure 10.1** Primordial follicle to secondary follicle formation (gonadotrophin independent phase): The oocyte, under the stimulatory effects of retinoic acid, recruits squamous type granulosa cells (GCs) to form the primordial follicle. Steroid hormones inhibit the formation of primordial follicles. Under the activating effects of stem cell factor (SCF)/kit ligand, basic fibroblast growth factor (bFGF), bone morphogenetic proteins 4 and 7 (BMP-4 and -7), platelet derived growth factor (PDGF), leukemia inhibitory factor (LIF) and keratinocyte growth factor (KGF), the primordial follicle further develops into the primary follicle with accompanying morphological changes of the supportive cells, i.e. from a monolayer of squamous type GCs to cuboidal type GCs. Thecal cells then surround the now multiple layers of GCs which encloses the developing oocyte to form the secondary follicle. Repressive signals like phosphatase and tensin homolog (PTEN), Foxo3 and anti-Müllerian hormone (AMH), tuberous sclerosis complexes (TSC) 1 and 2 and FOXL2 inhibits the development of primordial follicles to secondary follicles. See plate section for color version.

proliferate. These drops of follicular fluid coalesce to eventually form the follicular antrum.

Follicular antrum formation and antral expansion are absolutely dependent on FSH [68, 69]. The commencement of GC differentiation occurs upon follicular antrum formation, which corresponds approximately to the end of the oocyte growth phase. Granulosa cell differentiation produces two anatomically and functionally distinct lineages – mural GCs that line the wall of the follicle with primarily a steroidogenic role and the CCs, which encircle the oocyte [57, 70]. Adequately functioning CCs are essential for ensuring the survival and subsequent maturation of the oocyte, allowing it to fulfill its reproductive role [71].

As the pre-antral follicle develops into the antral follicle, its GCs are not only sensitized to FSH but also respond to LH directly, since they express both FSH receptors and LH receptors (LHR) at this time [72, 73]. Luteinizing hormone receptors are known to be located on the mural GCs but not on the CCs or the oocyte [74–76] during the antral phase. Oocytes do not possess functional gonadotrophin receptors but depend on follicular somatic cells to relay cues via the cumulus-derived microvilli projecting through the zona pellucida into the ooplasm [75]. The LH-dependent stages of oocyte and CC maturation are critically affected by paracrine signaling, most notably involving GDF-9 and BMP-15 [77]. Growth differentiation factor 9 is anti-apoptotic in pre-antral follicle and protects GCs from undergoing programmed cell death by activating the PI3K–Akt pathway [78]. Insulin-like growth factor-1 (IGF-1) also activates PI3K–Akt and has been shown to play an anti-apoptotic role in rat and bovine GCs by sustaining PI3K–Akt signaling. In humans, where IGF-2 is more abundant, it appears to act similarly to IGF-1 [78].

The WNT/β-catenin (CTNNB1) pathway is also known to have profound effects on the proliferation, differentiation and survival of GCs [79]. It enhances FSH-mediated induction of an aromatase gene (CYP19A1) in GCs, and its regulation of this gene appears to involve direct interaction with the transcription factor steroidogenic factor 1 (NR5A1). Decreasing CTNNB1 levels, or disrupting the

interaction with NR5A1, reduces FSH-induced CYP19A1 promoter activity and mRNA accumulation [80]. This affects estradiol synthesis and may adversely affect follicular development.

Expression of other components of the WNT/β-catenin pathway, including DVL, AXIN, GSK-3β and β-catenin, are also found in human CCs [81]. Beta-catenin is a key effector of WNT/β-catenin signaling and is regulated by the cytoplasmic destruction complex formed by AXIN, GSK-3β and APC. In addition, WNT2 and its receptor FDZ9 have also detected in human CCs. These findings are compatible with a model in which WNT2 signals through FDZ9 to regulate the β-catenin pathway in human CCs, potentially recruiting β-catenin into the plasma membrane and promoting the formation of adherens junctions CDH1 [81]. This may ensure the establishment of effective communication and proliferation of the cumulus oophorus in order to support the oocyte's development.

Another player which may have a role in follicular maturation is MATER (Maternal Antigen That Embryos Require). It is expressed in CCs but not in mural GCs [82]. It is transcribed only during oogenesis and remains stable until the morula and early blastocyst stage but disappears in the late blastocyst stage [83]. It is demonstrated to interact with protein kinase C epsilon ($PKC_{\xi}$) in human CCs. As $PKC_{\xi}$ is believed to function as an anti-apoptotic protein, it is possible that MATER–$PKC_{\xi}$ collaborates with other signaling pathways such as PI3K, Akt and Ras/Raf/ERK to regulate cell survival and cell death. Therefore, a proper MATER expression during follicular maturation could be crucial for pro-survival signal transduction such as via the PI3K/Akt/ $PKC_{\xi}$ pathways [84].

After the acquisition of LH receptors by GCs, subsequent follicular maturation comes under direct LH control. Estrogen secretion is sustained through LH support of GC aromatase activity and thecal androgen synthesis, augmented by paracrine inhibin [52, 73]. Luteinizing hormone-driven nutritional and information signals from CCs promote oocyte quality [85, 86], and the oocyte in turn secretes BMP-15 and GDF-9 to influence CC expansion, apoptosis, carbohydrate metabolism and steroidogenesis in GCs [77, 87].

These molecules presented here are probably part of the myriad of factors ensuring the survival and growth of GCs to sustain follicular development. Without the support of gonadotrophins on somatic cells, the oocyte will fail to mature and the entire follicle will undergo atresia (Figure 10.2).

## Antral phase and resumption of meiosis

As the follicle enters into the antral phase (which lasts about 8–10 days), the antral cavity becomes filled with follicular fluid and continues to grow reaching a diameter of 10–12 mm [7]. The antral follicle with GCs and TCs, which can bind LH when the LH surge occurs, is highly likely to be the "dominant" follicle which persists to become the pre-ovulatory follicle (measuring up to 25 mm in diameter). During this phase of follicular development, it is imperative for the oocyte, arrested at diplotene stage (known as the germinal vesicle), to resume meiosis within the next 30–36 h, allowing a meiotically competent oocyte to be released at ovulation.

Stimulation of meiotic maturation by LH occurs via its action on the surrounding somatic cells rather than on the oocyte itself [12]. The resumption of meiosis is characterized by the disappearance of the nuclear membrane, a process referred to as germinal vesicle breakdown (GVBD).

Cyclic adenosine monophosphate (cAMP) is a negative regulator of GVBD [88]. A constitutively activated G protein-coupled receptor, GPR3, is responsible for generating cAMP required for maintaining meiotic arrest during oocyte development [89]. Also, cAMP-dependent protein kinase A (PKA) has been shown to be required for meiotic arrest in oocyte, as knocking down of the regulatory Iα (RIα) subunit of PKA results in resumption of meiosis in mouse oocytes with abnormal meiotic spindles and cleavage planes leading to extrusion of large polar bodies [90]. The cAMP-dependent PKA regulates the activity of maturation promoting factor (MPF, also known as Cdk1/cyclin B1) by phosphatase Cdc25 and kinase WEE1/MYT1 [91]. The MPF is inhibited by inactivation of Cdc25 (through phosphorylation by PKA) and phosphorylation of Cdk1 of MPF by WEE1/MYT1 (activated by PKA) [12].

Gonadotrophins promote an increase in cAMP levels in GCs and a decrease of cAMP in oocyte, thus inducing the resumption of meiosis as well as cumulus expansion [92, 93]. Activation of mitogen-activated protein kinase (MAPK) by a notable surge of cAMP in the cumulus oocyte complex, caused by

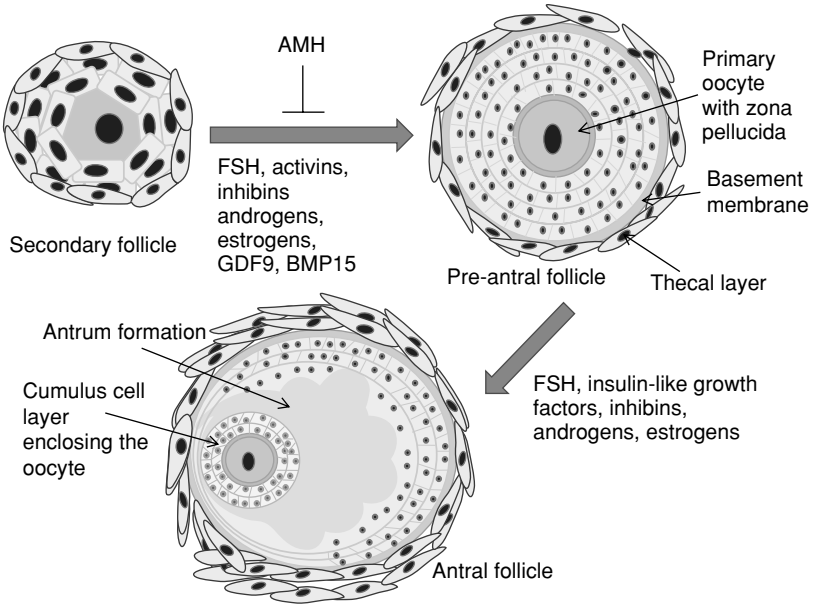


**Figure 10.2** Pre-antral phase to antral phase transition. As the secondary follicle develops, it becomes more gonadotrophin sensitive with the expression of gonadotrophin receptors. Under the activation of follicle stimulating hormone (FSH) and the effects of activins and inhibins, androgen synthesis with estrogen production take place, resulting in the development of the secondary follicle into the pre-antral follicle. Anti-Müllerian hormone (AMH) has been shown to suppress early stages of follicular growth and onset of responsiveness to FSH in vitro, exerting a controlling influence on the rate at which follicles become available for pre-ovulatory development. Enhancing effects from growth differentiation factor (GDF-9), bone morphogenetic protein 15 (BMP-15) and insulin-like growth factors enables the pre-antral follicle to develop further into the antral follicle with the formation of the antrum. At the same time, the GCs proliferate and differentiate into the cumulus cell layer which encloses the developing oocyte. With the antral cavity filled with follicular fluid, the pre-antral follicle now becomes the antral follicle. See plate section for color version.

stimulation with gonadotrophins, triggers the resumption of meiosis as well as cumulus expansion [94]. The MAPK appears to mediate LH-induced oocyte maturation by interrupting cell-to-cell communication through phosphorylation of connexin-43 within the ovarian follicle [95]. The breakdown of communication arrests the supply of cAMP from somatic cells to oocyte, thereby reducing the levels of cAMP within the oocyte [96]. This is probably mediated by termination of gap junctions between CCs and the oocyte [97]. The rapid increase of cAMP in CCs may activate phosphodiesterase 3A (PDE3A) and decrease cAMP level in oocytes, possibly by cAMP-dependent guanine nucleotide exchange factor (GEF)/PI3K/phosphoinositide-independent protein kinase 1 (PDK1)/protein kinase B (PKB) pathway [98, 99].

Following the LH surge, a cascade of events is initiated that leads to CC proliferation. The competence to undergo expansion is a unique characteristic of CC differentiation [100], which has been shown to be critical for normal oocyte development, ovulation and fertilization [101–103].

Nitric oxide and/or natriuretic peptides-derived cyclic guanosine monophosphate (cGMP) may be an oocyte maturation inhibitor. The cGMP maintains pre-ovulatory oocytes in meiotic arrest via inhibition of oocyte cAMP phosphodiesterases (e.g. PDE3) to maintain cAMP level and activation of cGMP-dependent protein kinase (PKG) to decrease MAPK activity [94]. It is possible that accumulation of cGMP by nitric oxide (NO) and/or natriuretic peptides under FSH stimulation during follicular growth may serve to prevent untimely oocyte maturation. The inducible form of nitric oxide synthase (main source of NO in the ovary) [104] significantly decreases after human chorionic gonadotropin (hCG) injection (a similar effect to the LH surge), which induces a decrease of NO concentrations in pre-ovulatory follicular fluid [105]. This results in a decrease of cGMP which may allow the oocyte to undergo maturation and ovulation.

Luteinizing hormone-binding to mural GCs leads to the production of epidermal growth factor (EGF)-like growth factors, such as amphiregulin, epiregulin and betacellulin [106]. These factors are known to be potent stimulators of oocyte maturation and they also activate genes like Has2, Ptgs2 and Tnfaip6 associated with CC expansion [107]. Epidermal growth factor receptor (EGFR) activation by EGF or EGF-like growth factors may be a common pathway mediating meiosis-inducing influence of FSH and LH. The EGFR is expressed in both CCs and the oocyte, whereby the EGFR in CCs contributes significantly to the mediation of gonadotrophin-induced meiotic resumption [94]. The pathway utilized by EGF and EGF-like growth factors for the stimulation of meiotic resumption involves binding to EGF receptor subfamily members with subsequent tyrosine kinase activation and downstream activation of MAPK [99]. Possible production of steroids, e.g. progesterone, estradiol by EGFR and the PI3K/PKB pathway, mediates gonadotrophin-induced MAPK activation [94].

Sterols and steroid hormones have also been shown to result in the resumption of meiosis. Follicular fluid meiosis-activating sterol (FF-MAS), an intermediate in the cholesterol biosynthetic pathway, is a putative oocyte maturation-inducing substance, demonstrated to stimulate resumption of meiosis in isolated mammalian oocytes including mouse, rat and humans [108–112]. Addition of FF-MAS to culture medium promotes progression of the oocyte to metaphase II and dramatically improves the quality of oocytes produced in vitro [113–115].

Progesterone is produced in human CCs [116]. The level of progesterone and its receptors in CCs are increased by stimulation with LH and FSH in porcine cumulus oocyte complexes [117]. Progesterone biosynthesis depends on the gonadotrophin-induced activity of its biosynthetic enzymes such as CYP51, delta 14-reductase, delta 7-reductase, P450scc and 3βHSD [118, 119]. Progesterone may induce GVBD by binding to its receptor, which then decreases connexion-43 in CCs and, hence, reduction of cAMP level in oocytes [120, 121]. However, the role of estrogen and testosterone in mediating mammalian oocyte remains to be elucidated, and it seems that steroids are not necessary for resumption of mammalian meiosis. Nevertheless, steroids are probably involved in follicular growth, somatic cell-differentiation and the acquisition of developmental competence of mature ova [94, 122] (Figure 10.3).

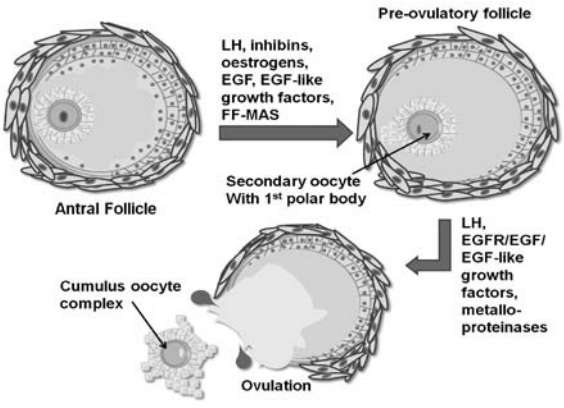


**Figure 10.3** Antral phase to ovulation: The antral follicle progresses to develop into the pre-ovulatory follicle under the stimulation of luteinizing hormone (LH). This transition is augmented by epidermal growth factors (EGF), EGF-like growth factors and follicular fluid meiosis-activating sterol (FF-MAS). A critical step in this transition is the completion of meiosis I in the maturing oocyte with the extrusion of the first polar body. This ensures that the oocyte achieves meiotic competence prior to ovulation. Under the LH surge and increased expression of metalloproteinases and proteolytic enzymes, the pre-ovulatory follicle subsequently ruptures and ovulation takes place with the release of the cumulus oocyte complex. The follicle then collapses and proceeds to form the corpus luteum. See plate section for color version.

## Ovulation and subsequent luteinization of the follicle

Luteinizing hormone appears to play a major role in mediating the final phase of follicular development. Under LH stimulation, there are significant changes in the pattern of steroid secretion from primarily aromatizing androgens to estrogens to synthesizing progesterone. The somatic cells have reduced capacity to bind estrogens and FSH, but become sensitized to LH and respond by secreting more progesterone. This prepares the pre-ovulatory follicle for ovulation, depresses the growth in other less mature developing follicles and promotes the transition to the luteal phase of the cycle [7].

By the end of the pre-ovulatory phase, the follicle attains a maximum dimension of ~25 mm in diameter, mostly due to the rapid expansion of follicular fluid volume. This results in the oocyte with its associated CCs to be held tenuously by a thinning stalk of mural GCs to the rest of the follicle. Under the LH surge, the oocyte resumes meiosis, extrudes the first polar body and arrests at metaphase II. Activation of the Mos/MAPK signaling pathway seems to be the

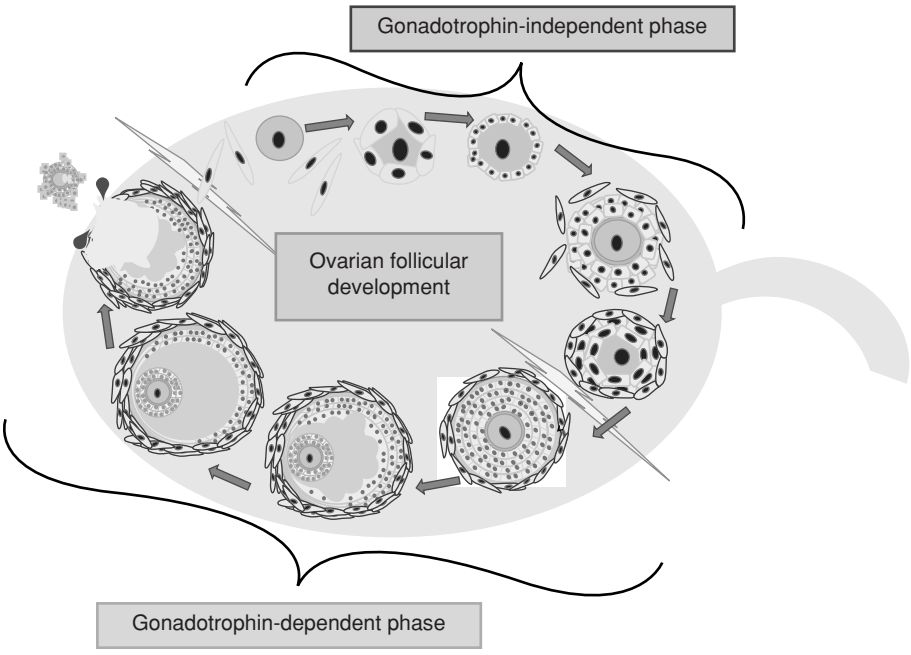


**Figure 10.4** In the ovary, follicular development constitutes of a gonadotrophin-independent and a gonadotrophin-dependent phase. Coordination of multiple growth factors, hormones and biochemical molecules signaling at timely intervals with the activation of several pathways, e.g. PI3K, JAK/STAT, WNT/β-catenin and MAPK between the growing oocyte, surrounding somatic cells and wider endocrine system ensures optimum follicular development with the release of a meiotically and developmentally competent oocyte. See plate section for color version.

cytostatic factor responsible for this second meiotic arrest [123]. At the time of ovulation, the oocyte becomes basically transcriptionally inactive [124, 125]. From this point until the 4–8 cell stage (2–3 days after fertilization), the oocyte/embryo must survive on stockpiled mRNAs and proteins.

Prior to ovulation, due to increased vascularization of the follicle, the follicle appears hyperemic. There is production of large amounts of prostaglandins (mainly $PGE_2$) and synthesis of a hyaluronan (HA)-rich matrix. These events are dependent on LH-induced expression of EGF-like factors (amphiregulin, epiregulin, betacellin) [106, 126], matrix-associated molecules (Has2, Ptgs2, Tnfaip6, Ptx3, Cspg2), transcription factor genes (Pgr and RUNX1) and protease genes (Ctsl and Adamts1). Ovulation seems to be reminiscent of inflammatory responses due to the genes expressed under LH stimulation [127]. The LH surge also induces a switch in gene expression in surrounding somatic cells and the generation of an extracellular matrix within the expanded cumulus oocyte complex.

The EGFR transactivation has been demonstrated to be essential for the regulation of ovulation [126], and the physiological surge of LH requires a local sustained activity of the EGFR to mediate and maintain its stimulation in the GCs which synchronizes the many complex events that finally converge to ovulation [128].

Matrix metalloproteinases (e.g. gelatinase), serine proteases such as plasmin and plasminogen activator (which cleaves pro-collagenase to generate active collagenase) are explicitly involved in ovulation as proteolysis is essential to effect the breakdown of the connective tissue of the follicle. The LH surge leads to activation of these proteases to cause follicular rupture and the release of the cumulus oocyte complex.

After ovulation, the collapsed follicle transforms into the corpus luteum. The fibrin core within the follicular antrum undergoes fibrosis over a period of few days and the membrane propria between the granulosa and theca layers break down and blood vessels invade. Luteinizing hormone is pertinent in terminating GC proliferation and mediates the genetic transition of GCs to luteal cells (LCs) [129]. The GCs will cease dividing and hypertrophy to become large lutein cells rich in mitochondria, smooth endoplasmic

reticulum, lipid droplets and, in many species, a carotenoid pigment, lutein, which may give the corpus luteum a yellowish or orange tint. Thecal cells will form smaller lutein cells and produce progesterone and androgens. This transformation of GCs and TCs to lutein cells is referred as luteinization.

In humans, the luteal phase lasts from 12 to 15 days. Thereafter, luteal regression occurs with ischemia and the progressive death of lutein cells leading to the formation of a whitish fibrous scar known as corpus albicans. A PTEN deficiency in LCs seems to lead to enhanced phosphorylation and activation of Akt, and expression of a distinct set of PI3K pathway components including FOXO3. This appears to enhance longevity in LCs [130]. Hence, PTEN appears to be expressed in a cell-specific manner in the ovary, as described previously with its involvement in the oocyte, GCs at various stages of development and in the regulation of LCs' life span.

Due to the regression of the corpus luteum, there is a fall in progesterone output. A new cycle then reinitiates in the ovary with the recruitment of primordial follicles and progression of subsequent events as detailed before.

## Concluding remarks

Follicular development consists of gonadotrophin-independent and gonadotrophin-dependent phases (Figure 10.4). These two phases require the fine coordination of multiple growth factors, hormones and biochemical molecules signaling at timely intervals between the growing oocyte, surrounding somatic cells and the wider endocrine system. The precise control and balance of these systems is essential if the ovulation of a meiotically and developmentally competent oocyte is to be achieved.

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Chapter 11

# Fundamental cryobiology of reproductive cells and tissues

## Concepts and misconceptions

Erik Woods, Sreedhar Thirumala, Xu Han and John K. Critser

## Introduction

Cryobiology as a science deals with the effects of reduced temperatures on living organisms, their constituent parts and their products. Understanding the basics of cryobiology to develop improved cryopreservation procedures has been a major challenge to scientists all over the world. Since the complexity of cryobiology is deeply rooted in the complexity of living systems, historically this has entailed coordinated research efforts among biological and physical sciences and involved the participation of biologists, chemists, physicists, engineers, mathematicians and others using carefully designed empirical evaluations, as well as investigation and utilization of specific cellular characteristics in theoretical models, all in efforts to apply cryopreservation to a broad range of cells and tissues [1].

For the most part, reproductive cryobiology has been developed to gain time, either for flexibility (e.g. extension of fertility), logistics (e.g. gamete donation), back-up (for repeat embryo transfer) or some combination of these [2].

Many misconceptions related to the cryopreservation or vitrification methods stem from the use of potentially confusing terminology that has evolved in cryobiology literature. At one level, this occurs in the labels we place on various categories of general methods for cryopreservation. For example it is common to refer to methods on the basis of cooling rate such as "slow cooling methods" or "rapid cooling methods" [3–5]. In other cases cryobiologists refer to methods as "equilibrium methods," "non-equilibrium methods" or even "quasi-equilibrium methods" [6]. Still, in other cases the methods are described by the type of container or device in which the cells or tissues are cryopreserved such as an "Open Pulled Straw" method [7], a "needle-immersed vitrification" method [8], a "Cryoloop" method [3] and so on.

The key to sorting-through what all this means in terms of outcomes is to understand how these "labels" (methods) relate to the underlying physiochemical conditions produced that fundamentally determine whether cells and tissues survive (or fail to survive) the series of extreme conditions we subject them to during the cryopreservation process. For the purposes of organizing this chapter and to potentially clarify misconceptions and/or misuses of terminology with respect to cryobiological processes, the subject has been divided into three parts consisting of: (1) classical cryopreservation; (2) vitrification; and (3) preservation of reproductive cells and tissues using classical cryopreservation and vitrification procedures.

## Classical cryopreservation

Cryopreservation has been developed to store viable biological systems at ultra-low temperature (–196°C) in a cryogenic medium such as liquid nitrogen for extended periods of time. At such ultra-low temperatures, all cellular divisions and metabolic activities are arrested, such that the systems can be revived and restored to the same living state as before they were stored. Most currently used methods for cryopreservation of mammalian cells are indeed derived from centuries of low temperature work on reproductive cells and tissues [9].

Cells and tissues lose viability (are killed) during classical cryopreservation due to two major causes: (1) the formation of ice inside the cells (intracellular

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ice formation or IIF); and (2) exposure to very high concentrations of solutes that form as liquid water is removed from the extracellular solution when it crystallizes and forms ice (solute damage) [10]. In general, these factors are managed by following "equilibrium" cryopreservation methods where "equilibrium" means that the water inside and outside the cells is in equilibrium (or very near equilibrium). It is important to understand that equilibrium cryopreservation simply means that the procedure is intended to maintain (more or less) the same amount of water inside the cell as outside the cell. All the other components of the system (the cryopreservation medium and the cytoplasm) are typically not in equilibrium.

During a typical cryopreservation process ice tends to form at different rates. The generally accepted theory of ice development in biological systems is based on Mazur's work, which states that the probability of an ice crystal to form at any temperature is a function of volume [11]. As the extracellular space is much larger than the intracellular space, ice is likely to form first in the extracellular space whereas intracellular solution becomes supercooled. Water is removed from suspending solution due to this ice formation, and partially frozen extracellular solution becomes rich in solutes. This creates an osmotic non-equilibrium state that provides a driving force for the cellular dehydration or loss of intracellular water [12]. This phenomenon is a function of temperature, cooling rate and initial amount of solutes dissolved in the water. At low cooling rates cellular dehydration is the dominant mechanism while at very rapid cooling rates intracellular ice formation (IIF) is the dominant mechanism [11, 12]. Both excessive cellular dehydration and IIF have been shown to be deleterious to the post-thaw survival of biological systems [10–13]. At sufficiently slow cooling rates the cell loses water to concentrate the intracellular liquid adequately enough to eliminate supercooling and maintain chemical potential of intracellular water in equilibrium with that of extracellular water. However, the resulting changes in the extracellular space, such as changes in pH, and/or changes in ionic concentration, can lead to protein configuration changes, modifying their properties and diminishing or eliminating biological activity. This denaturing of cell proteins due to solute toxicity may be lethal to cell survival [13]. In addition, mechanical interaction between extracellular ice crystals and cells can also lead to the physical deformation of cells and rupture of cell membranes [14]. On the other hand, if the cell is cooled too rapidly it is not able to lose water fast enough to reach equilibrium; it becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly. Intracellular ice formation is generally thought to be lethal as it causes injury to cellular membranes and intracellular structures. So, cooling rates which are either too slow or too fast can and do reduce the post-thaw survival of the cells; therefore, a cooling rate for maximum cell cryosurvival should and does exist between the "high" and "low" rates [15]. This has been confirmed experimentally for a variety of cells and the curve of cell survival, plotted as a function of the cooling rate, has a characteristic inverted U-shape [10]. The use of cryoprotectants helps to alleviate some of the problems associated with solute toxicity and IIF during freezing.

## Equilibrium and non-equilibrium slow freezing

Conventional slow freezing protocols involve pretreatment of cells with cryoprotective agents (CPAs) in order to remove some water from the cells and to minimize some other harmful effects of freezing. Broadly speaking, CPAs can be divided into two classes including permeating (e.g. polyols such as glycerol or ethylene glycol) and/or non-permeating (e.g. sugars). Permeating CPAs are thought to protect via their colligative properties [1] while non-permeating CPAs are generally thought to protect by dehydration as well as membrane stabilization [1]. While both types of CPAs result in some cellular dehydration, the majority of cell water does not exosmose until slow cooling in the presence of ice to a predetermined temperature. During freezing the fate of any biological cell depends on the thermal history it experiences, i.e. cooling rate, end temperature to which the cell is frozen, the time spent at the end temperature and warming or thawing rate [16].

During slow freezing, using low concentrations of CPA, IIF is generally avoided by cooling cells sufficiently slow so that dehydration reduces the intracellular water at near osmotic equilibrium with the outside partially frozen solution. This type of slow freezing is referred as "equilibrium slow freezing" which avoids the supercooling of intracellular solution and averts the formation of lethal IIF. In equilibrium, freezing cells are typically cooled at 0.1–1.0°C/min using programmable freezer to a temperature of –60°C or below. The presumption is that after reaching –60°C, the cells

essentially contain little or no freezable water and consequently IIF cannot occur during a subsequent plunge at –196°C [17]. The first successful cryopreservation of mouse embryos using equilibrium slow freezing was reported by Whittingham *et al.* and Wilmut and Rowson [18, 19]. These authors reported a high percentage of embryo survival when frozen at ≤1°C/min to –70°C under near-equilibrium conditions. However, extreme dehydration, as would be encountered during equilibrium slow freezing, can cause volume excursions (shrinkage) beyond what some cells can tolerate and produce irreversible damage to cellular components. Additionally, as described above, unavoidable excessive salt concentrations can also play a significant role in damaging the cells.

Alternatively, if the cooling rate is not slow enough to maintain equilibrium, IIF becomes possible due to supercooling of intracellular solution in relation to the frozen extracellular solution at a given temperature. However, there is an alternate slow freezing approach often referred as "non-equilibrium slow freezing" or "interrupted slow freezing" [20] during which the cells are initially cooled slowly (slow enough to avoid IIF) to an intermediate temperature to render some degree of dehydration. At such an intermediate temperature, it is expected that the concentration of unfrozen fraction of the suspending solution and cytosol are significantly high enough to enable vitrification and form amorphous glass upon rapid cooling by immersing abruptly in liquid nitrogen. However, high survivals with this procedure usually require that the cells be warmed rapidly during thawing. The reason for this is that after the initial slow cool to the intermediate temperature, the cells still probably contain a small amount of freezable water and that water undergoes damaging recrystallization unless the subsequent warming is rapid [17]. Further, if the concentration of unfrozen fraction is not enough an unstable glass may form during rapid immersion in liquid nitrogen and devitrification and/or re-crystallization can occur upon re-warming [9].

## Toxicity and osmotic effects of cryoprotectants

In general, classical cryopreservation (as well as vitrification) involves the addition of permeating CPAs, and their extensive use is based on the serendipitous observation by Polge *et al.* that sperm cells were able to survive freezing in the presence of a chemical (glycerol) and did not survive the freezing process in its absence [21]. Another ground-breaking finding was the utility of dimethyl sulfoxide (DMSO) as a CPA by Lovelock and Bishop in 1959 [22]. Over the course of some 50 years, several other compounds have been found that possess some CPA activity and many cells and tissues have been frozen, mostly through empirically derived methods. The literature shows that there is a considerable divergence in classes of CPAs, varying from low molecular weight permeable solutes like DMSO, glycerol, ethylene glycol and sucrose [13, 23] to high molecular weight non-permeable polymers like polyvinylpyrrolidone (PVP), dextran and hydroxyl ethyl starch [23, 24]. While these compounds have enhanced cryopreservation outcomes and made widespread use of cryostorage possible, there is a substantial amount of evidence suggesting that CPAs, albeit with their benefits, can actually play a direct role in producing cryoinjury [23, 25, 26]. Detrimental effects of cryoprotectants are almost as relevant to cryobiology as are their cryoprotective effects [25]. For instance, the CPAs can potentially induce osmotic injury to the cells during their addition and removal, and the higher the CPA concentration required, the greater the likelihood of damage [1]. While permeating CPAs do indeed penetrate cells, none to date have been identified that cross their membranes as quickly as water. Addition of a permeating CPA therefore causes the cell to undergo extensive initial dehydration due to osmotic efflux of water followed by rehydration due to influx of CPA and water. During removal of CPA, cells at first swell due to the osmotic influx of water and then slowly return to initial isotonic volume as CPA and water leave the cell. These repeated volumetric changes can result in significant loss of functional integrity and even cell death [27].

The rate at which permeable CPA diffuses into the cells varies between the cryoprotectants and is also temperature and concentration dependant [28, 29]. For example, most embryos are more permeable to propylene glycol than to glycerol, which renders them less sensitive to osmotic shock when propylene glycol is diluted out compared to glycerol [28]. For this reason, impermeable solutes such as sugars are often added to dilution media to prevent excessive osmotic swelling during post-thaw CPA removal. As mentioned earlier, CPA diffusion across the membrane is also a function of concentration and temperature. The higher the concentration, the faster and more

extensive the osmotic shock. This may be overcome either by gradual or stepwise addition and dilution of CPAs before freezing and after warming [1]. Temperature has a profound effect since the permeation of CPA is usually rapid at higher temperatures. In reproductive cells such as embryos, CPA permeation essentially ceases when temperature reaches around –5°C [28]. Cryoprotective agents also reduce the temperature at which ice crystallization first occurs, thereby extending the dehydration time during freezing. Therefore, procedures for the addition and removal of CPA must be optimized according to specific cell characteristics to ensure successful cryopreservation. The dynamics of cell volume changes can be maintained within the tolerable limits by carefully selecting the optimal type and concentration along with a cooling rate that yields the optimum time necessary for diffusion of CPA in and out of the cell during cooling in the presence of ice. For this, information is required regarding the osmotic tolerance limits of cells, defined as the extent of volume excursions cells can withstand before irreversible loss of function occurs [30]. In addition to that, to optimize addition and removal of CPA it is also necessary to define other osmotic properties such as osmotically inactive cell volume, hydraulic conductivity and solute permeability of the cell [31].

## Vitrification

As described in many recent research papers and reviews, vitrification has become an increasingly accepted method for preserving embryos, oocytes and, recently, even sperm [2]. However, despite the growing popularity of this type of preservation, misconceptions in the literature abound. Vitrification may be simply defined as the process of converting a supercooled liquid into a glass-like amorphous solid which is free of any crystalline structure, either by the quick removal or addition of heat and/or by mixing with an additive. Vitrification occurs at the glass transition temperature ($T_g$) which is lower than the melting point ($T_m$) [32].

In the context of cryobiology, the supercooled liquid is almost always water and the additive is a combination of solutes, typically including salts and one or more permeating or non-permeating CPAs. The relationship between "the quick removal of heat" (i.e. cooling rate) and total additive (solute) concentration can be expressed conceptually as:

$$P_V \approx (S \times P)^B;$$

where $P_V$ is the probability of vitrification, S is the total solute concentration, p is the specific solution parameter (derived empirically), and B is the cooling rate.

Vitrification prevents both intracellular and extracellular ice formation [33]. One approach to vitrifying cell suspensions is to use a relatively high concentration of CPA in combination with a relatively low cooling rate. In this case, the cooling rate must be higher than the critical cooling rate (CCR) required to achieve vitrification [34–36].

To determine the CCR, Ren *et al.* [36, 37], based on Boutron's [34, 35] semi-empirical crystallization theory, developed a correlation between the cooling time and the volume ratio ($x$) of the ice quantity to the maximum crystallizable ice by using the integral method without considering the finite expansion. This correlation can determine CCRs through investigations on the time-temperature transformation (TTT) diagram and its derivative continuous-cooling transformation (CCT) diagram [35–38]. For solutions with a relatively high concentration of CPAs, such as 4–5 M glycerol, L-2,3-butanediol and 1,2-propanediol, CCRs were determined as $10^{3\sim4}$ K/min [36]. Currently available cooling methods, such as the Open Pulled Straw (OPS) method [3, 7, 39, 40], can indeed achieve cooling rates that high. However, as previously described, high concentrations of CPAs often have damaging toxic and/or osmotic effects on cell survival [33]. Another approach to achieving vitrification is to use an ultra-fast cooling rate ($10^{5\sim6}$ K/min) to improve vitrification tendencies and decrease the CPA concentration requirement [41]. Critical cooling rates for solutions with a relatively low concentration of CPAs (such as 1∼2 M) may reach $10^{5\sim6}$ K/min [34]. Producing such ultra-fast cooling rates is important for vitrification of these solutions. In addition, previous investigations [34, 35] have also demonstrated that a crystallization peak exists as a temperature region typically from 240 to 200 K, where the maximum ice formation happens during freezing. This region is also a dangerous temperature region (DTR) for vitrification of a CPA solution [41]. The ultra-fast cooling rate should also significantly decrease the time for the sample to pass the DTR during cooling and hence to improve vitrification tendencies.

A common misperception held by many in assisted reproductive technology (ART) laboratories is that plunging samples into liquid nitrogen ($LN_2$) results in cooling rates sufficiently high to produce vitrification. To gain a better understanding of why this is a

misperception, it is helpful to consider the relationship between the heat transfer coefficient (h) of a sample, and how this is related to cooling rate, the size of the sample and the required CPA concentration in the sample to achieve vitrification.

The heat transfer coefficient comes from Newton's law of cooling: the dissipation of heat from a solid to a fluid is proportional to the temperature difference between the solid and the fluid. This can be expressed as:

$$q = hA(T_s - T_a);$$

where q is the heat flow, h is the heat transfer coefficient, A is the surface area, $T_s$ is the surface temperature, and $T_a$ is the reference temperature [42]. Using this approach we can show the relationship between the heat transfer coefficient (h) of a sample, and how this is related to cooling rate, the size of the sample and the required CPA concentration in the sample to achieve vitrification at various cooling rates. Plunging samples into $LN_2$ is an example of a pool boiling approach. Flowing $LN_2$ over a sample is an example of a forced flow boiling approach. Oscillating heat pipe (OHP) technology [41] is an example of new technologies that are being developed to increase our ability to apply vitrification approaches to biological samples (Figure 11.1 [41]).

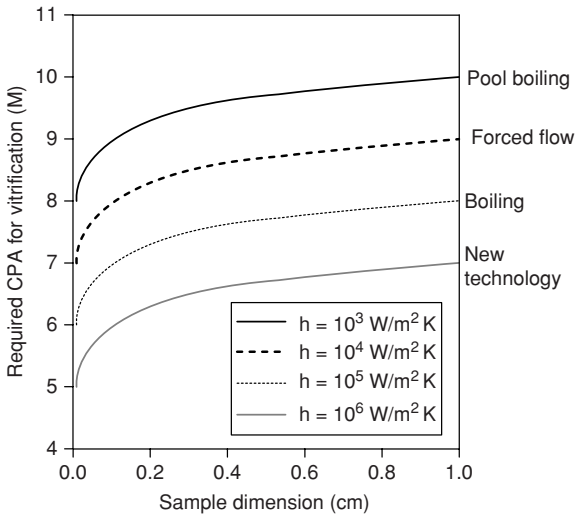


**Figure 11.1** The effect of sample size on the cryoprotectant agent (CPA) concentration required to achieve vitrification at various cooling rates. Plunging samples into liquid nitrogen ($LN_2$) is an example of a pool boiling approach. Flowing $LN_2$ over a sample is an example of a forced flow boiling approach. Oscillating heat pipe (OHP) technology (data from Jiao *et al.* [41]) is an example of new technologies that are being developed to increase our ability to apply vitrification approaches to biological samples. See plate section for color version.

## Equilibrium and non-equilibrium vitrification methods

As described above, in the context of cryopreservation, equilibrium refers to the relative amounts of water inside the cell and outside the cell being the same (or nearly so). An ideal vitrification method produces no ice formation and may therefore be an equilibrium method. If a sufficiently high concentration of CPA could be added at the beginning of freezing, formation of ice would be totally avoided and the system would vitrify with no supercooling no matter how slowly it was cooled. The lengthy isothermal equilibration in cryoprotectant solution can be represented by a long isothermal (near isothermal) streak on the phase change diagram before the system is cooled to glass transition temperature without ice crystallization (EV-1 on Figure 11.2 [43]). This approach, which is independent of cooling rate with no ice crystallization, may be called "equilibrium vitrification." It should be noted that, in equilibrium vitrification, post-thaw survival is not dependent on the rate of warming as there is no ice to re-crystalize. Using this approach, Song *et al.* vitrified vascular grafts by exposing them to 55% cryoprotective solution and then cooling them rapidly (~43°C/min) to –100°C followed by slow cooling (~3°C/min) to –135°C [44]. In another study, Brockbank *et al.* [45] exposed porcine cardiac tissue to an 83% v/v cryoprotectant solution followed by similar cooling to –135°C with good structural preservation. However, the concentration of CPA necessary to achieve equilibrium vitrification is extremely high (typically 60–80%) and it is often a daunting task to balance the deleterious effects of toxicity associated with such high CPA concentration with potential benefits. Earlier, Farrant introduced a liquidus tracking method, in which he gradually increased CPA concentration while lowering the sample temperature during freezing and gradually removed CPA during rewarming in order to minimize the known toxic effects of the cryoprotectant [46]. By this method, the sample remained slightly above the melting temperature on the phase change diagram and effectively cooled below glass transition temperature without any supercooling (EV-2 on Figure 11.2). Farrant explored his technique using smooth muscle tissue but, despite his early success in avoiding ice crystal formation, the

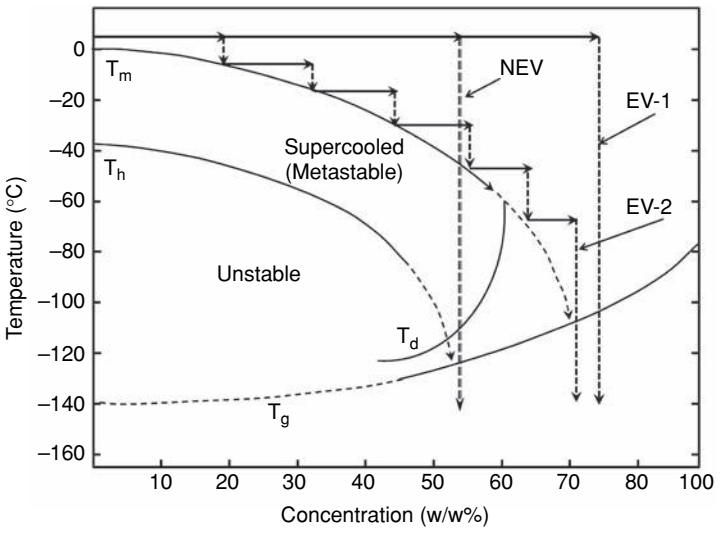


**Figure 11.2** Binary phase diagram for aqueous mixtures of glycerol showing the principal events and phase changes associated with cooling. Where $T_m$ is equilibrium melting point curve; $T_h$ is homogeneous nucleation curve; $T_d$ is devitrification curve; $T_g$ is glass transition curve; EV-1 is the equilibrium vitrification by the liquids' tracking method; EV-2 is the equilibrium vitrification by warm equilibration in unfreezable solution and NEV is the non-equilibrium vitrification. Adapted from Anderson [43].

rewarmed tissue was severely impaired both functionally and structurally after exposure for 20 h in unfrozen CPA medium at –79°C [46]. Subsequent experiments by Elford and Walter determined that the toxic effects of DMSO are not solely responsible for the loss of tissue function, and in fact its function was only improved after adequate steps were taken to optimize the solute concentration (the ionic composition) of the CPA medium during freezing [47]. Nevertheless, Farrant's eminent procedure has been the basis for most of the equilibrium vitrification methods that followed [48–51]. Despite some success achieved, this equilibrium approach has not been actively pursued, presumably because the technique requires lengthy periods of exposure to toxic solutes at high temperatures and, more importantly, the equilibration at sub-zero temperatures may not be achievable either in a practical time scale or without exceeding the tolerance limits of solute toxicity of the tissue [47, 52].

Vitrication is also possible using less concentrated cryoprotective solutions if sufficiently rapid cooling rates are employed. This approach requires the best possible cryoprotective concentration (critical CPA concentration) that ideally balances the glass formation ability and toxicity at the temperature of addition [48]. These optimally adjusted cryoprotectants could be vitrified by supercooling at realistically feasible cooling rates through the metastable non-equilibrium zone between the melting temperature and glass transition temperature on the phase change diagram (NEV on Figure 11.2) [53]. The success of non-equilibrium vitrification depends on how fast the sample is cooled through the temperature region of potential crystallization to below glass formation temperature. Since the supercooled water exists in a state of precarious equilibrium, the cooling rate has to be rapid enough to extend supercooling all the way down to glass forming temperature; otherwise any minor perturbations or suspended impurities can trigger ice nucleation and crystal growth. In concentrated solutions, the probability of ice nucleation becomes high well below the freezing point, where the actual rate of ice crystal growth is significantly low [54]. Clearly, if the cooling rate is rapid, the sample can escape both nucleation and ice crystal growth and reach an amorphous glassy state. However, if the cooling rate is insufficient the sample may nucleate but with or without ice crystals. In the later case, if the warming rate is not sufficient, freezing reoccurs as the sample traverses the nucleation zone first and then the zone of ice growth as larger ice crystals grow at the expense of smaller crystals [54]. These phenomena are known as devitrification and re-crystallization, respectively, which are of great concern during non-equilibrium vitrification. Another factor that influences the critical CPA concentration required to achieve vitrification under moderate cooling conditions is the volume of the sample. Minimizing the volume of the sample decreases the amount of liquid which has to be cooled and the likelihood of ice crystal formation and thereby promotes vitrication [55]. Nevertheless, non-equilibrium vitrication has been developed and shown to provide

effective preservation for a number of cells, including monocytes [56], organized tissues [44, 48–51, 57], mouse ovaries [58] and pancreatic islets [59].

# Preservation of reproductive cells and tissues

## Cryobiology of spermatozoa

The benefits of sperm cryopreservation are numerous in human reproductive medicine. For example, ART using cryopreserved donor spermatozoa is a widely available treatment for couples with severe male infertility factor (such as oligospermia, seminoma or azoospermia) or for patients without male partners desiring pregnancy [43]. Another established option for semen cryopreservation is for men and post-pubertal boys who are suffering either from malignant diseases such as cancer or at risk of fertility impairment [60]. For instance, in patients suffering from cancer, the germinal epithelium of the testis, from which the spermatozoa develop, is highly sensitive to drugs, chemotherapy and radiotherapy treatments. Therefore, the patients are encouraged to cryopreserve their sperm before cured and sterilized by these treatments to preserve their ability to father a biological child in the future either using intrauterine insemination (IUI) or intracytoplasmic sperm injection (ICSI) [61]. Human sperm cryopreservation can also be used to preserve fertility in other situations, such as surgical procedures that can harm fertility and prior to vasectomy.

It is just over 60 years since Polge *et al.* described the use of glycerol to freeze spermatozoa, and several attempts have since been made to develop successful cryopreservation protocols for mammalian spermatozoa with varying success [21]. Cryopreservation of sperm, developed largely by empirical means, generally employs a slow-cooling rate (about 5°C/min) starting from physiological temperature to a seeding temperature, followed by rapid cooling next to the onset of ice formation (100–200°C/min), in the presence of glycerol buffered with egg-yolk citrate medium [1]. The problem of cryoprotectant toxicity and osmotic stress, as well as biochemical alteration and possible effects at the genetic level are widely investigated to determine optimal CPA addition and dilution, and cooling and warming rates [62, 63]. Various aspects of sperm cryopreservation, such as chemical composition of extenders and their effects on the sperm plasma membrane, osmotic tolerance limits, hydraulic conductivity and CPA permeability, seminal plasma composition and other factors that influence the quality and life-span of post-thaw spermatozoa have also been studied [63, 64]. Also, to better assess the success of cryopreservation, endpoints other than the percentage of motility recovery or the assessment of ultrastructural damage, like energy status, damage to the plasma membrane or to subcellular elements, chromatin stability and chromosomal damage have been proposed [65]. Many of the studies generated acceptable results in various species, but the procedures used are still relatively complicated and time consuming and considerable loss of recovery is still observed [1]. While, in humans this loss of viability may not be an issue with normal ejaculate volumes and sperm counts, they may be highly significant in the case of oligozoospermic or asthenozoospermic samples [62]. Furthermore, with the development of ICSI and the availability of techniques for surgical sperm retrieval (both epididymal and testicular), there is an increased need to store low numbers of sperm and therefore develop improved freezing techniques in order to maximize survival [62, 66]. Efficient cryopreservation of small number of sperm retrieved from these techniques reduces the number of surgical interventions and thus avoids the complications and expenses associated with repeated surgeries. Moreover, given the unique characteristics of epididymal and testicular spermatozoa, conventional methods of sperm cryopreservation may not be optimal [67].

Novel cryopreservation methods have been recently proposed to improve post-thaw recovery in highly compromised and low-number sperm samples. A novel procedure for efficient cryopreservation of single human spermatozoa in cell-free human or animal zona pellucida is reported [66]. Other novel methods include, using mini straw as carrier for cryopreservation of microquantities of sperm, direct cryopreservation of individually selected spermatozoa in microdroplets, the Cryoloop method using conventional slow freezing, the microencapsulation of sperm in alginate beads, using ICSI as a carrier for single sperm cryopreservation and agarose microspheres as analogue to zona pellucid for small sample sperm cryopreservation (refer to a recent review by AbdelHafez *et al.* [67]). In all these methods, sperm was pre-equilibrated in CPA solution before cooled either using $LN_2$ vapor or controlled rate freezer and then immersed in $LN_2$ [67]. Despite their novelty and

attractiveness, these methods invariably suffer from several drawbacks such as labor intensive, high cost, extreme complexity, sperm loss due to adherence to carrier walls, risk of cross contamination and sperm loss during washing off the carrier.

Recently, vitrification methods have been gaining momentum in sperm cryopreservation. Generally vitrification involves a very high concentration of solutes to ensure total vitrification without any ice crystal formation. While quite successful for several types of reproductive cells, this method of vitrification is inappropriate for the cryopreservation of mammalian spermatozoa due to extreme sensitivity of spermatozoa to permeating CPA [68]. However, several studies have shown success in achieving vitrification of sperm samples at moderate to zero CPA concentrations. For example, Schuster *et al*. demonstrated success by using Cryoloops for ultra-rapid freezing of a small number of human sperm with 12% glycerol as CPA in test-yolk solution [69]. In their pioneering work, Dr. Isachenko's group from Germany have successfully attempted to vitrify a small volume of sperm without any cryoprotectants by using different carrier systems such as Cryoloops, droplets, OPS and grids [68–73]. They demonstrated that vitrification of a small sample of spermatozoa without cryoprotectant resulted in higher motility after rapid warming in comparison to conventional freezing with cryoprotectant [68]. Thus, it appears that intracellular vitrication can be achieved at relatively low cooling rates without any CPAs and preliminary dehydration. This is most probably facilitated by the low intracellular water content and the presence of abundant high molecular weight components such as proteins, polysaccharides and nucleic acids in spermatozoa, which affect the viscosity and glass transition temperature of the intracellular cytosol [71, 72].

The success rate of slow-freezing cryopreservation of spermatozoa has been, for the most part, satisfactory [1]. Nevertheless, because of the damage associated with freezing, the motility of post-freeze spermatozoa is statistically reduced with respect to pre-freeze motility, and success rates vary among species and even among individuals within species. This associated loss of viability may not be an issue where ejaculate volume and sperm counts are normal. However, in situations where the sperm retrieval yields extremely smalls samples, conventional slow freezing may not be suitable and other appropriate methods including vitrification may be considered. Finally, although CPA-free vitrication offers a fast and simple method relative to slow freezing, the current evidence is not sufficient to support the use of vitrification as standardized protocols for semen cryopreservation. To make any rational conclusions on this, well-designed randomized trials with appropriate sample sizes are needed to evaluate the effectiveness of various semen-freezing methodologies.

## Cryobiology of oocytes

Cryopreservation of mammalian oocytes is one of the most intensively studied topics in the field of cryobiology. For example, due to their regular spherical shape and relatively large size, mouse oocytes were used as model cells for numerous theoretical investigations and the tests of newly developed cryopreservation methods. Human oocyte cryopreservation plays not only an important clinical role in assisted reproduction as an adjunct to sperm and embryo cryopreservation, but also a representative of the application of both the freezing and vitrification approaches. Therefore, in this section, the discussions are concentrated on the current status of human oocyte cryopreservation.

### Mature human oocytes

In the mature oocytes, the metaphase chromosomes are lined up by the thin meiotic spindle along the equatorial plate. Such spindle apparatus is fragile and can be easily damaged by IIF or cell volume change due to osmotic effects. The first report of a pregnancy and subsequent delivery of a baby derived from a frozen and thawed oocyte appeared in 1986 and 1988, respectively [74, 75]. Several other reports appeared in the late 1980s describing additional attempts to cryopreserve human oocytes [76, 77]. One notable feature of these reports is the lack of fundamental experiments designed to understand the cryobiology of human oocytes. Instead, simple changes to a standard equilibrium protocol were made and outcomes were assessed. Changes included altering the addition and removal of the cryoprotectant (stepwise and at room temperature [77]) and assessing the effects of the polyol propylene glycol (PG) versus DMSO [76]. In general, the outcomes of these early reports were poor and highly variable. For example, immediate survival of 136 oocytes recovered after thawing was 32% [77]; 58% of those that survived underwent fertilization and 2 pregnancies ensued but neither went to term. In the report by Al-Hasani *et al*., 28% of the oocytes frozen in dimethyl

sulfoxide ($Me_2SO$) survived and only half of those fertilized [76]. Oocytes frozen in PG tended to survive better (32% and 75% of those fertilized). The second report of a live birth came in 1987 using an equilibrium method with DMSO as the permeating CPA [78].

Since 2001, at least 10 reports have appeared describing the results of freezing human oocytes using a standard equilibrium method with 1.5 M PG and sucrose. Some of these reports show very high survival and early development rates, and this has lead some individuals to emphatically defend human oocyte cryopreservation and suggest that the clinical results are nearly equivalent to the use of fresh embryos. Other investigators have been more cautious in their interpretation [79], and in two recent reports with a very large number of cycles, the results were still rather poor [80, 81], especially when compared to the use of fresh embryos [81].

Vitrification has also been utilized as a means to cryopreserve human oocytes in recent years, gaining more popularity than traditional equilibrium methods in recent years. Kuleshova *et al*. described a birth resulting from an oocyte vitrified with a solution containing ethylene glycol and sucrose [82]. For this work, the investigators utilized an OPS for the procedure. Since this time, other reports on human oocyte vitrification have been published [83, 84], with all of these reports utilizing open container systems to achieve so-called "ultra-rapid cooling." However, the use of open containers represents a potential problem for the possibility of disease transmission in the storage vessel [85]. On average, the results from the vitrification trials have been better than the trials using slow cooling (as measured by the number of oocytes to achieve a pregnancy). However, the results are still limited in number and general conclusions are difficult to reach at this time. Overall, progress on human oocyte cryopreservation has been significant during the past few years, as evident by the number of reports appearing in the literature. However, the procedure is still regarded as suboptimal and experimental in nature.

### Immature human oocytes

Due to the absence of a metaphase II spindle in GV stage oocytes, it has been suggested that immature oocytes may be more amenable to cryopreservation [86]. Several reports have been published describing attempts to cryopreserve GV stage human oocytes. A low sodium solution proved more effective with GV oocytes compared to a standard sodium chloride-based solution, with similar rates of maturation, fertilization, and cleavage compared to controls [87]. In a different study, the maturation rate and fertilization rate of cryopreserved and control oocytes was similar, but blastocyst development was lower [88]. Vitrification has also proved successful with GV human oocytes [89], with rates of maturation, fertilization and early development similar between frozen and non-frozen oocytes. Despite these successes, to date, only one report describing a live birth after cryopreserving a GV stage human oocyte appeared in the literature [90]. Clearly, more basic research needs to be undertaken to improve this technology.

### Ovarian tissue

Successful ovarian tissue cryopreservation is one of the effective clinical options for preserving female fertility. For example, orthotopic re-implantation of cryopreserved ovarian cortical strips is a promising technique for restoring ovarian function in women treated with sterilizing chemotherapy for cancer. Both equilibrium freezing and vitrification methods have been applied for human ovarian tissue cryopreservation. Hovatta *et al*., using histological assessment, showed that a high proportion of follicles could survive an equilibrium-freezing method with human ovarian tissue [91, 92]. In the first report, they showed very good morphological survival of ovarian follicles after cryopreservation using either DMSO or PG, with no obvious differences between frozen–thawed and non-frozen tissue. In the second report, using an in-vitro culture system, they present data suggesting that follicle development occurs in the previously cryopreserved tissue, with development rates similar between the frozen–thawed and unfrozen tissue samples. Shortly after these reports, a study undertaken to determine the diffusion rates of cryoprotectants into human ovarian tissue was published [93]. The results from this study suggest that ethylene glycol (EG) and DMSO diffuse into the tissue more rapidly than PG and glycerol. These results supported the previous findings of these authors which showed EG and DMSO to be superior cryoprotectants compared to PG and glycerol [94], suggesting that the rate of cryoprotectant permeation is a critical factor in successful cryopreservation of human ovarian tissue. A similar conclusion was reached in a different report, where the time of equilibration prior to cooling had a significant effect on the proportion of intact follicles post-thaw [95]. Therefore, it is of both practical and theoretical

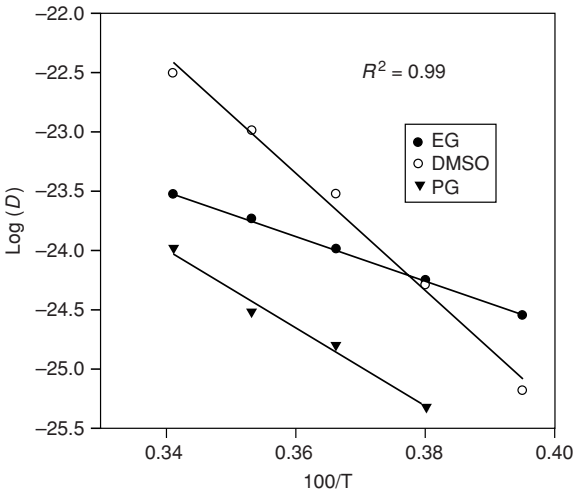


**Figure 11.3** The Arrhenius relationship between the values of $\bar{D}$ (unit: $m^2/s$) and the measurement temperatures (20, 10, 0, –10 and –20°C). DMSO, dimethyl sulfoxide; EG, ethylene glycol; PG, propylene glycol.

importance to address the issue of optimizing cryoprotectant permeation into human ovarian tissue by measuring permeability parameters of the relevant cells and using this information to model the mass transport of water and cryoprotectant. Various physical models regarding the permeation of cryoprotectants into tissues have been established. A differential scanning calorimetry method to measure the cryoprotectant–water mutual diffusivity ($\bar{D}$) in ovarian tissues at both super and sub-zero temperatures was also established [96]. Figure 11.3 shows that the Arrhenius relationship was strictly followed for the values of $\bar{D}$ with different cryoprotectants. Due to the relatively low values of $\bar{D}$ ($<10^{-6}$ $cm^2/s$), it has been suggested that the optimal cooling rate for equilibrium freezing procedures for ovarian cortical strip should be at the order of <0.5 K/min. For the cryoprotectant perfusion procedure before cooling, a stepwise perfusion is preferred and the perfusion time should be controlled to approximately 1 h.

Follicle loss after transplantation is likely due in part to ischemic damage [97–99]. However, using fresh or frozen ovarian tissue from cynomolgus monkeys, Schnorr *et al.* showed that only two of four recipients had functional frozen and thawed transplants, yet five of six of the ovarian transplant recipients receiving fresh tissue had functional transplants [100]. In a more recent study using ovarian tissue from cynomolgus or rhesus macaques, immediate post-thaw viability of follicles isolated from the thawed tissue showed a fairly high percentage of viable follicles (67–70%) with the various freezing methods used. This number was lower, however, when compared to follicles isolated from fresh tissue (76%). Due to the historical lack of established methods for the in vitro culture of isolated follicles [101], accurate assessments of the developmental potential of follicles after freezing has been difficult to establish.

In more recent years, several studies have shown that ovarian follicles can develop in previously frozen and thawed human ovarian tissue after xenotransplantation into immunodeficient mice. These reports showed the development of antral follicles [102], MII stage oocytes [103] and corporea lutea with increased levels of circulating progesterone [104, 105]. Despite these positive signs, the developmental potential of the oocytes from those follicles remained uncertain. At least 12 reports describing ovarian graft function after autotransplantation in humans have been published in the past several years (see Donnez *et al.* [106] for a recent review). In many of these studies, follicular development and accompanying endocrine changes, suggesting restoration of graft function were noted. However, to date, only two live births have been described [107, 108] resulting from transplantation of previously frozen ovarian tissue; in two other reports, pregnancies ended in miscarriage [109, 110].

## Cryobiology of embryos

Because of its central importance, the cryopreservation of human embryos has become an integral part of almost every ART program. According to International Committee Monitoring ART (ICMART), the majority of the children (up to 40% worldwide) born after ART are now born from cryopreserved embryos [111], and this number is likely to increase in future. Embryo cryopreservation is also a widely used method of fertility preservation for cancer patients [112]. Cryopreservation allows the storage of excess viable embryos for future use in an in vitro fertilization (IVF) treatment cycle. In addition, cryopreservation makes feasible the postponement of embryo transfer in the event of a mother becoming ill or with patients at high risk of ovarian hyperstimulation syndrome [113].

Over the years, major empirical advances have been made to develop successful protocols for the cryopreservation of embryos. Literature shows that majority of the studies either used glycerol, EG or

DMSO as CPAs. Recently, however, these cryoprotectants have been increasingly replaced by 1,2 propanediol, which is generally used in combination with other permeable and non-permeable cryoprotectants. Propanediol is considered to have higher penetration permeability and is less toxic than DMSO. Both slow cooling and vitrification have been studied for embryo cryopreservation. The slow cooling technique was developed independently in 1972 by Wilmut and Rowson for bovine embryos and Whittingham *et al.* for mouse embryos using DMSO as CPA [18, 19]. The same freezing protocol was applied to human early-cleavage stage embryos and resulted in the first report of pregnancy in 1983 [114] and live births in 1984 [115]. In 1985, PG along with sucrose was first introduced in human embryo freezing and proved to be quite efficient for 1, 2 or 3 day-old embryos [116]. Therefore, this method was rapidly applied and has become the widespread application for embryo preservation in virtually every full scale IVF program worldwide [117]. In general, slow freezing of embryos employ progressive pre-equilibration in a CPA solution and then cooling to a seeding temperature at which deliberate ice nucleation is initiated to prevent supercooling. After equilibration for about 10–15 min, the cells are cooled slowly ($<1^{\circ}$C/min) to about –30°C till they are sufficiently dehydrated and then finally plunged and stored in liquid nitrogen. Normally, frozen embryos are thawed slowly to prevent excessive osmotic stresses. Finally, the CPA is removed either by successive dilution or by using a sucrose dilution technique [118].

Slow cooling, however, is a long process and requires extensive resources like controlled rate freezers and large quantities of liquid nitrogen. Further, the studies using slow freezing have reported significant differences in survival and developmental rates after warming between laboratories, developmental stages and quality [119–121]. Vitrification, on the other hand, has increased greatly in use in recent years particularly for freezing reproductive cells, and avoids the multihour long process of slow cooling to –196°C. For vitrification, embryos are usually loaded with high concentrations of CPA and then plunged directly in $LN_2$ using some specialized carrier or supporting device. For thawing, usually high warming rates are used to prevent ice crystallization during devitrification. There are, however, several factors that need to be taken into consideration while undertaking the vitrification of embryos. The type of CPA used, its concentration and exposure time, all create toxicity issues. These can be alleviated to some extent by using combinations of permeating and non-permeating CPAs or by loading embryos with CPAs at sub-zero temperatures [1]. There are also some concerns regarding contamination in $LN_2$ tanks as open carrier systems allows direct contact of embryos with $LN_2$ posing a risk for disease transmission and rendering their use in human IVF debatable [122].

Nonetheless, despite some drawbacks, vitrification has become a viable and promising alternative to traditional slow freezing, and previous published data appears to indicate that vitrification produce at least equal or significantly better results than those obtained from slow cooling for cryopreservation of mammalian embryos [122, 123]. For example, Kuwayama *et al.* compared slow freezing with vitrification by using human embryos at various stages of development [122]. They reported that vitrification resulted in 100% survival of pronuclear stage embryos with 93% cleavage stage rate and 52% blastocyst rate. Conversely, with slow freezing the pronuclear stage survival rate, cleavage stage rate and blastocyst rate were only 89, 90 and 41%, respectively. They further demonstrated that vitrification was superior to slow freezing for cryopreservation of four-cell embryos and blastocysts. However, clinical pregnancy rates after the transfer of frozen thawed embryos were not significantly different between the two methods of cryopreservation [123]. On the other hand, Rama Raju *et al.* reported significant difference in pregnancy rates of 35% and 17.4% after vitrified and slow-cooled 8-cell embryo transfer, respectively [124]. In a study by Li *et al.*, randomized cryopreservation of 160 day-3 embryos resulted in no significant difference in post-thaw survival (vitrification: 89% versus slow freezing: 91%) and clinical pregnancy rates (vitrication: 48% and slow freezing: 38%) [125].

Although it appears that vitrification is superior to slow freezing in terms of post-thawing survival rates in different developmental stages of human embryo cryopreservation, it is premature to make any solid conclusions regarding the relative efficacy of vitrification versus slow freezing. This is mainly due to the fact that, at present, there are not enough randomized control test data available [2]. Therefore, additional randomized controlled trials using uniform criteria for defining post-thaw embryo quality are needed to determine if vitrification should be the preferred method of embryo cryopreservation in IVF laboratories.

## Conclusion

Within the realm of reproductive cryobiology, a better understanding of the basic features of what we have described here as "classical cryopreservation" and "vitrification" is critical to accelerate advancement and to allow individual clinical laboratories to make processing choices appropriate to reaching their desired outcomes. This includes clarification of terms, theoretical and technical details, and a balanced, pragmatic evaluation of possible risks and potential (or definite) gains [2]. As the survey of preservation methods and results for gamete, tissue and embryos presented here indicates, the ability to preserve these cells and tissues has had a profound effect on humankind with millions of births around the globe facilitated by these efforts while truly "optimum" protocols are yet to be uncovered. Because of the complex nature of the biological systems as well as the physical phenomenon in question, a scientific approach balanced between theoretical and empirical methods is absolutely necessary to ensure procedures continue to be further optimized.

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Chapter 12

# Fundamental aspects of vitrification as a method of reproductive cell, tissue and organ cryopreservation

Steven F. Mullen and Gregory M. Fahy

## Introduction

In the field of assisted reproductive technology, vitrification is becoming an increasingly popular method of cryopreserving cells, tissues and even entire organs [1–4]. The National Library of Medicine's public database (PUBMED) lists an exponentially increasing number of citations on vitrification over the past decade, many of them pertaining to vitrification in reproductive biology.

Vitrification in the present context is the process whereby an aqueous solution is transformed into an amorphous solidified system as a result of a significant decrease in temperature [5]. The three-dimensional arrangement of the molecules in such a system resembles that of a liquid (i.e. remains disordered), but the viscosity and associated shear relaxation time are more solid-like. This is in contrast to freezing methods, where molecular rearrangements in the form of extensive ice formation occur in the solution during the cooling process.

Vitrification as a means of cryopreservation is an attractive alternative to freezing for several reasons. The two most obvious are the complete elimination of ice formation and its consequent damage, and the ability to avoid chilling injury by rapidly cooling a sample through the temperature zone known to be damaging [6–8]. Other advantages include reduced labor and equipment costs [9]; the lack of need to discover optimum cooling and warming rates; and scalability from cells to tissues and even to whole organs. Some recent reports suggest that vitrifying oocytes and embryos is a more effective means of cryopreservation than is freezing [10–13], providing additional support for its adoption in a clinical setting.

The formation of ice during a cryopreservation procedure is often associated with damage to the biomaterial [14, 15]. More than 30 years ago, intracellular ice formation (IIF) visualized using cryomicroscopy was shown to be strongly correlated with irreversible damage to mouse oocytes [16]. While examples of non-lethal IIF are found in the literature [17–19], it is currently believed that, in most cases, IIF is lethal [14] and should be avoided. But even extracellular ice formation can cause significant damage, whether by acting to nucleate intracellular ice [20] or by disrupting the extracellular matrix of organized tissue or compressing packed cells [21]. Perhaps most significantly, survival after cryopreservation by freezing is the result of a compromise in which some cells may die due to IIF and some may die due to excessive shrinkage, often making it difficult to achieve 100% survival. Vitrification eliminates this dilemma. Hence, it is reasonable to expect that the complete avoidance of ice formation during cryopreservation would improve outcomes.

A discussion of the specific methods of vitrification currently utilized in fertility preservation and the rationale for those methods will be covered by other chapters in this volume. The purpose of this chapter is to provide a brief historical overview of vitrification as a means of cryopreservation; to delineate some of the principles governing crystallization, vitrification and storage in the vitreous state; and to discuss strategies for developing improved vitrification solutions. Given that the current emphasis in reproductive cryobiology has been to come as close to freezing as possible without actually allowing ice to form (in order to be able

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to use the minimum possible concentrations of cryoprotectants), some understanding of the physics of ice formation is necessary to understand the risks associated with different vitrification methods and, indeed, to be able to determine whether vitrification is complete or partial. Interested readers are encouraged to consult a number of authoritative reviews for further details [3–5, 9, 22–29].

## Historical origins of vitrification as a means of cryopreservation

The possibility that water might be vitrified was first proposed by Brayley in the mid 1800s [30], but the idea of cryopreservation by vitrification was apparently not introduced until Stiles observed, in 1930, that protoplasm is likely, at very high cooling rates, to form "a finely crystalline or even amorphous mass" that "in thawing, might be expected to give again the original system without change" [31]. It took Father Basile J. Luyet, however, to independently develop this idea into a major research proposal in 1937 [32]. Luyet's rationale for vitrification was that life is "due to a special and exceptional arrangement of the atoms, or other structural elements of living cells" [33] and that death from freezing "seems to result from the disruption of the units which constitute living matter when the molecules of water are torn away from these units by the forces of crystallization" [34]. This problem would clearly be prevented by vitrification, and Luyet observed that "the essential problem of the vitrification technique consists in … obtaining a cooling velocity sufficient to prevent the formation of crystals" [32].

Luyet and his colleagues focused on this problem until about 1958 [24]. In that year, perhaps inspired by criticism of Luyet's evidence for vitrification by Audrey U. Smith in 1954 [35], two fateful articles were published in *Biodynamica*, one by Luyet and Rapatz and the other by Meryman. These papers showed that gelatin gels previously believed by Luyet to be vitreous in fact contained ice in the form of "evanescent spherulites" that were detectable both optically [36] and by X-ray diffraction [37]. This revelation abruptly ended Luyet's pursuit of vitrification, and in 1969 he described his results as "mostly negative" and "of academic interest" [38]. Thus, Luyet introduced vitrification as a concept, but he did not provide it as a workable cryopreservation method that was widely adopted by others. He did, however, continue his studies in other areas beyond 1958, focusing on ice crystal morphology and phase diagram relationships rather than on the pursuit of vitrification. Ironically, it is these later studies that ultimately provided the physical basis for vitrification as it would later develop [24].

In 1978, Boutron and Kaufmann recognized that "in the extreme case of a solution which remains entirely amorphous even at very slow cooling or warming rates, all cells should be protected" [39]. However, they also rightly noted that the high concentrations required for vitrification tend to be toxic [40], but lower concentrations result in the need for astronomical warming rates to escape from freezing during re-warming [39], a less than encouraging situation. Although he ultimately obtained survival of putatively vitrified red blood cells in 1984 [41], the need to warm these cells at 5000°C/min and their lack of DNA did not make a particularly inspiring general case for vitrification, and this limited Boutron's early impact on the practical development of vitrification as a new and general method of cryopreservation. However, Boutron's seminal contributions to our understanding of the cooling and warming rate dependence of ice formation, and therefore of glass formation and stability of the amorphous state during warming, remain unparalleled (see discussion below and Mehl [27]).

Fahy's trajectory toward vitrification began in 1970, when he read of the partial success of Barry Elford in recovering smooth muscle strips that were prevented from freezing at temperatures as low as –79°C by using very high concentrations of dimethyl sulfoxide ($Me_2SO$) to depress the freezing point [42]. Fahy pursued this concept as an undergraduate student using frog sciatic nerves [43], and found that the nerves could tolerate 30% but not 40% $Me_2SO$ [G. M. Fahy, unpublished results]. As a graduate student he found that he could treat rabbit renal cortical slices with 40% $Me_2SO$ at –22°C with reasonable recovery, but not with 50% $Me_2SO$ at –35°C [44]. His inquiries into this approach continued when, as a postdoctoral fellow pursuing the goal of organ cryopreservation, he learned that, according to unpublished data of Rajotte and McGann, dog kidney cortical slices could survive exposure to 30% $Me_2SO$ plus 30% sucrose, an impressively high total concentration of cryoprotectant [45]. However, he soon found that solutions of this kind required total concentrations of 70–80% (plus 5% w/v glucose added to simulate a carrier solution) to prevent freezing for long periods at –79°C, and most solutions either froze or developed ice spheres millimeters in diameter (Table 12.1).

**Table 12.1** Supercooling properties of solutions containing sucrose.[a]

| % Glucose | % Sucrose | % Glycerol | % $Me_2SO$ | % EG | % MeOH | Result[b] |
|---|---|---|---|---|---|---|
| 5 | 40 | 30 | 0 | 0 | 0 | F |
| 5 | 40 | 30 | 0 | 0 | 5 | F |
| 5 | 30 | 44 | 0 | 0 | 0 | F |
| 5 | 30 | 40 | 0 | 0 | 0 | F, NF |
| 5 | 30 | 40 | 0 | 0 | 5 | IS |
| 5 | 30 | 40 | 0 | 5 | 5 | NF |
| 5 | 30 | 35 | 0 | 0 | 10 | IS |
| 5 | 30 | 30 | 0 | 0 | 10 | F |
| 5 | 30 | 30 | 10 | 5 | 5 | NF |
| 5 | 20 | 30 | 10 | 5 | 5 | IS |
| 5 | 20 | 30 | 20 | 0 | 0 | IS, NF |
| 5 | 20 | 25 | 25 | 0 | 0 | IS |
| 5 | 20 | 20 | 20 | 0 | 0 | F |
| 5 | 20 | 20 | 20 | 5 | 0 | F |
| 5 | 20 | 20 | 20 | 10 | 0 | IS |

[a] %w/v glucose and sucrose, % v/v glycerol, $Me_2SO$, EG and MeOH. EG, ethylene glycol; MeOH, methanol. Experiments carried out from 12/21/1979 to 3/17/1980; previously unpublished data.
[b] Results determined after 11–19 days of storage in triplicate ~15ml samples in test tubes. F, frozen; IS, ice spheres; NF, not frozen.

Although these results were discouraging, Fahy reasoned that success might still be obtained if the central problem of prolonging the time required for ice to form and grow in the deeply supercooled state could somehow be solved, and he reflected on how this might be done without using lethal solute concentrations. The idea of lowering the temperature below –79°C immediately led to a sudden inspiration: what if solutions of tolerable concentrations could be cooled all the way to the glass transition temperature without freezing? They would then be stable indefinitely! Thus was born the idea of achieving vitrification by the reliable deep supercooling of even organ-sized volumes into the vitreous state.

Fahy had previously seen that $Me_2SO$ solutions and pure ethanol remained transparent but fractured when cooled in liquid nitrogen ($LN_2$), so he began extensive and still-unpublished studies of fracture avoidance in 1980. These studies were soon followed by studies on reducing toxicity by the use of cryoprotectant mixtures [46], including putative toxicity-blocking amides [46–48], studies on the minimum concentration of cryoprotectant required for vitrification [48, 49], and the use of 1,2-propanediol [48, 49] and high hydrostatic pressures [48, 49] to reduce the latter. By mid 1981, he had assembled enough pieces of the puzzle to present the idea in public for the first time [48] and published confirmation of the utility of high pressures [50]. More thorough expositions of the idea were provided in 1982–1984 [22, 49, 51, 52].

By 1984, Fahy had clearly defined the physicochemical requirements for true vitrification; had demonstrated the possibility of highly concentrated yet minimally toxic solutions for vitrification; had explained the idea of combining permeating and nonpermeating cryoprotectants to induce vitrification in a manner consistent with the vitrification tendency of the intracellular compartment; had provided efficient methods for adding and removing vitrifiable concentrations; had described taking advantage of osmotic shrinkage for facilitating vitrification; had demonstrated methods for avoiding fracturing even in objects larger than rabbit kidneys; and had presented his new preservation method as one that should be applicable not just to organs but to all cells and tissues [22]. However, few cryobiologists concerned themselves with organ cryopreservation, and therefore few read about these developments or realized their broader applicability. In addition, there still remained no actual demonstration that living cells vitrified according to Fahy's methods could actually survive after re-warming.

Fortunately, William Rall became aware of Fahy's ideas and recognized the opportunity to make a major contribution by proving their applicability to mammalian pre-implantation embryos, a system of great interest to many cryobiologists then and now. Rall had himself concluded that slowly frozen embryos survive as a result of intracellular vitrification [53, 54], so he realized that embryos should be able to tolerate both intracellular and extracellular vitrification, making embryos an excellent system for an initial demonstration of Fahy's method. Rall joined Fahy's lab at the American Red Cross, and the two worked together to show that embryos could survive vitrification both at cooling rates applicable to small organs (~20°C/min, achieved by cooling at maximum speed in Fahy's small organ high pressure vessel) and at much higher rates. Fahy provided the vitrification solution, VS1, and Rall provided the method for adding and removing it.

The result of this collaboration [55] definitively established, for the first time, vitrification as a viable and potentially general alternative to freezing. Since then, the number of papers on biological vitrification has grown at an exponential rate [3, 56]. The systems that have been successfully preserved by vitrification to date are too numerous and diverse to list here, but include many examples from within the realm of reproductive biology, as reflected in the balance of this book, and finally even include, as of 2009, the survival and life support ability of the entire rabbit kidney following vitrification, re-warming and transplantation [57]. For additional details about the history of vitrification, the reader is referred to other partial accounts [3, 24, 45, 57, 58].

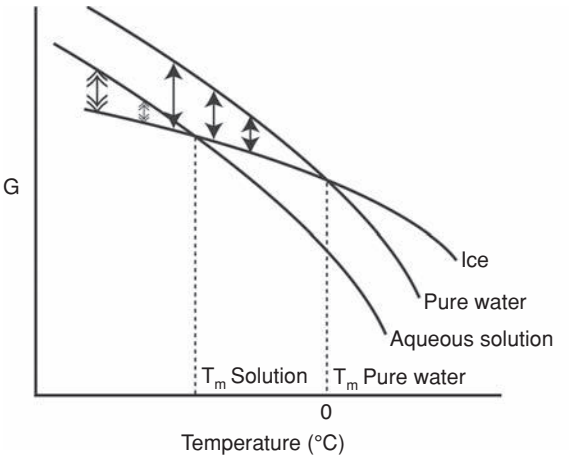


**Figure 12.1** An example of the relative values of G for pure water, water containing dissolved solutes and ice. A physical state is more thermodynamically stable than an alternative state if its value for G is lower than that alternate state's value. As can be seen, $G_{ice}$ is lower than $G_{pure\ water}$ at low temperatures; hence ice is the more favorable state ($\Delta G$ is negative). The temperature where these lines cross is the melting point of water ($T_m$ pure water; 0 °C), and at that point, both states are equally stable. Adding solutes to water causes the curve to shift to the left, decreasing the value of G relative to pure water. This decreases the temperature at which ice becomes the more stable state ($T_m$ solution). The curve for solutions would continually be shifted to the left if additional solutes were added, further depressing the melting point. The relative difference between the curves for water and ice at a specific temperature (as indicated by arrows) is an indication of the degree of supercooling for a solution remaining liquid below $T_m$, and can be thought of as a driving force for a phase transition. Hence, as a liquid is lowered below its melting temperature, the strength of the driving force continually increases. Note the difference in the length of the arrows for $\Delta G$ for water-ice (single arrowheads) and solution-ice (double arrowheads) at specific temperatures. Modified from Angell and Senapati [59].

# Physical aspects of vitrification

## Ice nucleation and growth

Whether water in aqueous solutions is thermodynamically stable in the liquid or solid phase at a specific temperature is determined by the difference in the Gibbs free energy (G) between each phase at that temperature [59]. As the temperature of an aqueous solution is lowered below its melting temperature ($T_m$), $G_{ice}$ becomes less than $G_{liquid\ water}$, and ice becomes the more stable state. $G = H - TS$, where G is a function of both the enthalpy (H; heat content) of a system and the entropy (S; degree of order), T being the temperature on the Kelvin scale. Both H and S decrease as the temperature is lowered; hence, whether the difference between the two states ($\Delta G$) is positive or negative depends upon the relative contribution of H and (–TS) to the overall energy of a system. Overall, G increases as temperature decreases for water in an aqueous solution, and the same is true for ice. However, $G_{liquid\ water}$ increases faster than $G_{ice}$, and it is this ever increasing difference in G for the two states that is the driving force for crystallization (Figure 12.1).

Despite $\Delta G$ ($G_{ice} - G_{liquid\ water}$) becoming negative as cooling passes below $T_m$, immediate crystallization rarely occurs. Crystallization is initiated by random and statistically improbable aggregations of water molecules to form small volumes of the new phase (known as ice crystal nuclei) [60]. Creation of a stable nucleus entails overcoming an energy barrier associated with the formation of the liquid–crystal interface. The interfacial energy of a nucleus is related to its

radius of curvature, with smaller nuclei having larger surface free energies per unit area. As a result, at temperatures slightly below $T_m$, only very large (and therefore statistically very improbable) nuclei are stable. In fact, in most instances, ice nucleation during cooling does not occur through the self-aggregation of water molecules to form a stable nucleus (a process called homogeneous nucleation), but rather takes place by water molecules becoming organized on foreign particles that effectively reduce the amount of ice–liquid interface and thus lower the free energy barrier associated with the formation of a stable nucleus. This process is termed heterogeneous nucleation, and the temperature at which this occurs is generally substantially higher than the homogeneous nucleation temperature ($T_h$). It is only at temperatures far below $T_m$ that homogeneous nucleation becomes energetically favorable. The homogeneous nucleation temperature of pure water is near –40°C [60]. It has been estimated to require 45 000 water molecules to form a stable nucleus at –5 °C, but only 70 molecules at $T_h$ [60].

Once a stable nucleus is formed, crystallization continues through crystal growth. Crystal growth is a kinetic phenomenon; molecules must diffuse from the liquid phase to the interface and rotate to be incorporated into the crystal. As a result, the crystal growth rate is highest at temperatures near $T_m$ where molecular mobility is high. Nucleation is also a kinetic process, and at lower temperatures the rate of molecular motion slows so much that the nucleus formation rate is reduced (Figure 12.2 [61]).

Based upon these ideas, it is easy to understand why the current methods for oocyte vitrification have evolved. Cooling very quickly minimizes the time available for nucleation and crystal growth to occur. Under the right conditions (see below) a solution can therefore be cooled and warmed rapidly with no apparent ice formation. Vitrification, however, does not inherently rely upon very high rates of cooling because ice nucleation and growth rates go down as solute concentration goes up. High cooling rates simply make vitrification more likely, and also, as discussed below, diminish the solute concentration necessary to attain a vitreous state.

## The necessity of the vitreous state

Vitrification can be seen as the means by which an aqueous solution remains within the bounds of thermodynamic law. As a solution is cooled to

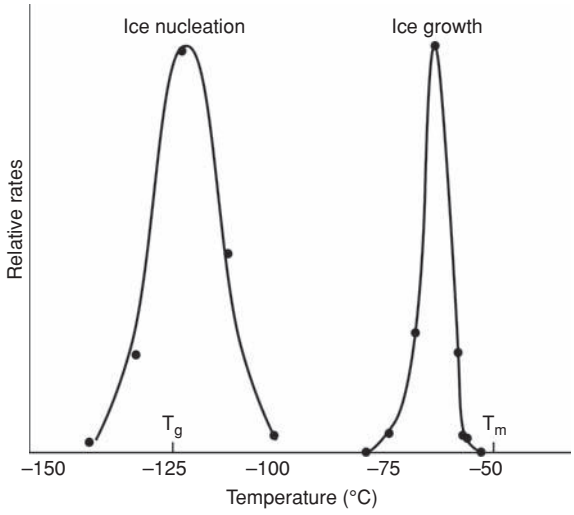


**Figure 12.2** The maximum rates of ice nucleation and ice growth occur at very different temperatures in effective vitrification solutions. The curves represent the temperature dependence of these two rates for a specific vitrification solution (M22) [58]. Whereas the maximum rate for nucleation occurs near $T_g$, the maximum rate for crystal growth occurs near $T_m$. With permission from Wowk [61].

temperatures below $T_m$, an interesting relationship between the entropy of the supercooled solution and the entropy of an identical solution undergoing freezing causes the entropy to plummet, but below $T_m$ the rate of reduction of the entropy of the supercooled liquid is greater than that of the corresponding frozen solution. If the trend were to continue to even lower temperatures, the supercooled solution would eventually have less entropy than the corresponding mixture of concentrated solution and ice. Such a situation, however, would be in violation of the known principles of thermodynamics because a crystal has the lowest entropy possible for a given substance. Kauzmann discussed this paradox and elucidated a rationale for its resolution [62].

With a sufficient reduction in temperature, thermal energy becomes insufficient to drive rotational and translational motions. Water molecules become trapped in local energy wells due to the removal of internal energy and the entropy therefore stabilizes, and remains above that of the ice phase. This event is referred to as the glass transition, and it occurs at temperatures between about –110°C [63] and about –130°C [39, 40] for low molecular weight cryoprotectants in water. The viscosity of a glass is around $10^{13}$ Poise, 15 orders of magnitude greater than the viscosity of water at room temperature!

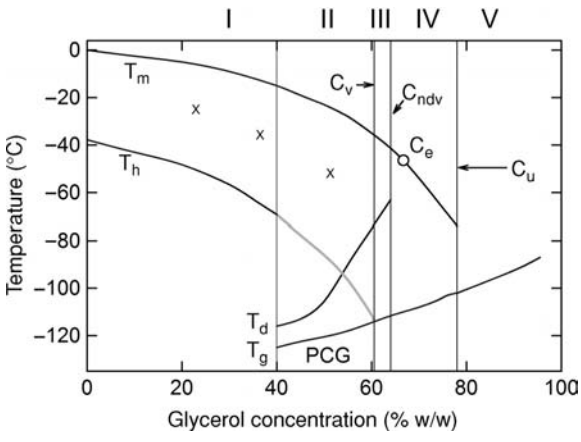


**Figure 12.3** Relationships between the values for the melting temperature ($T_m$), homogenous nucleation temperature ($T_h$), devitrification temperature ($T_d$), the glass transition temperature ($T_g$), the concentration needed to vitrify without homogeneous nucleation ($C_v$), the concentration allowing no devitrification ($C_{ndv}$), the eutectic concentration ($C_e$; glycerol usually does not crystallize during cooling) and the concentration that is sufficient to prevent the growth of pre-existing ice (thus, the "unfreezable concentration," $C_u$) for glycerol dissolved in water (modified from previous representations [3, 22, 49]). In Section I, even above $T_h$, ice nucleation events, indicated by Xs, are generally inevitable due to heterogeneous nucleation. On the contrary, in Sections IV and V, ice formation never occurs during slow cooling and warming. In Section II, sufficiently rapid cooling results in partially crystallized glass (PCG) due to the presence of at least homogeneous nuclei even though visual inspection would suggest the absence of ice. Section III defines the lowest concentration range for true vitrification. See text for more details.

## The concentration dependence of vitrification and devitrification

A supplemented phase diagram (Figure 12.3, for glycerol in water in this particular example) [22] is often used to visualize the relationships between key variables pertinent to vitrification. As can be seen by the curves in Figure 12.3, $T_m$ and $T_h$ both decrease with increasing solute concentration, and the glass transition temperature ($T_g$) increases. With enough solute, $T_h$ can be reduced to below $T_g$, making vitrification possible without contaminating homogeneously nucleated ice nuclei. Below this threshold concentration, $C_v$, one forms partially crystallized glass (PCG) unless cooling is so accelerated as to drive $T_h$ to lower temperatures such that $T_h$ again intersects $T_g$ at the concentration employed. Partially crystallized glass formed by rapid cooling with homogeneous nucleation is particularly liable to crystallization on warming to $T_d$, the temperature of devitrification (ice formation on warming).

Figure 12.3 is partitioned into sections, with each section bearing a particular relevance to cryopreservation. In section I, solute concentration is relatively low and therefore nucleation (via homogeneous and heterogeneous mechanisms) and extensive ice growth is inevitable [22] except at ultra-rapid cooling rates (see below). For solutions with concentrations associated with section II, crystallization during cooling may be minimal, but nucleation still occurs extensively. Such a solution may appear to be vitrified, and classification as such may be appropriate. However, such "doubly-unstable" glasses (unstable both thermodynamically and by virtue of being extensively nucleated) are prone to devitrification, i.e. extensive crystal formation and growth during warming [2, 5]. Such instances of crystallization may not be damaging if cooling and warming rates are high, for the size of individual crystals may remain relatively small, and the crystals may be in the form of cubic ice, which is believed to be more innocuous to biological systems than regular hexagonal ice [41]. At slightly higher concentrations (region III), homogeneous nucleation is at least nominally precluded due to $T_h$ being below $T_g$ ("nominally" because holding just below $T_g$ may allow the sub-$T_g$ extension of the $T_h$ curve to be observed [64]); the lower boundary of region III thus defines the minimum concentration needed to vitrify at slow cooling rates ($C_V$) [22, 49]. However, until the upper bound of region III, devitrification is still a problem. Beyond region III, even devitrification fails to occur during slow warming, making this the ideal region for vitrification. Unfortunately, for most cryoprotectant solutions, such concentrations are extremely toxic, and thus cannot be used successfully, although this problem is being overcome [58, 65].

It should be noted that the locations of these boundaries are not absolute due to the fact that the values of $T_h$, $T_d$, and $T_g$ are rate dependent. The values shown apply at cooling and warming rates of 10°C/min, but at extreme cooling and warming rates associated with some of the open systems utilized for oocyte vitrification, the point of intersection between $T_h$ and $T_g$ will be at lower concentrations. Thus, solutions containing lower concentrations of cryoprotectants can remain amorphous during vitrification with these systems. However, mapping phase diagrams at extreme rates is difficult, and to date we lack diagrams like Figure 12.3 that are applicable under such conditions.

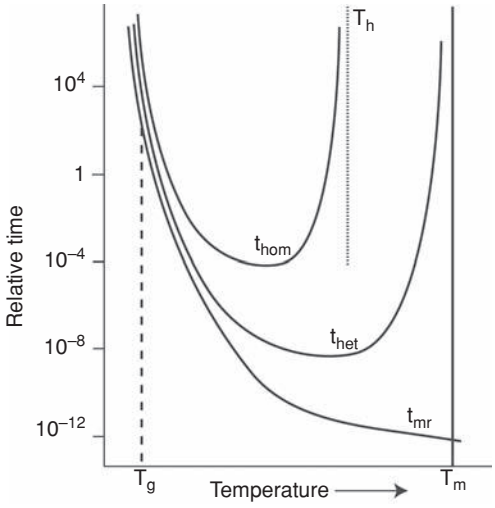


**Figure 12.4** Generalized time scales for a specific fraction of a solution to crystallize during cooling (via heterogeneous or homogeneous nucleation) as well as the time necessary for molecular rearrangements to occur within a solution ($t_{mr}$) as a function of temperature. At temperatures just below the melting temperature $T_m$, crystallization can occur, but is unfavorable due to the instability of small nuclei. With continued temperature reduction, nucleation occurs and the time for crystallization drops dramatically. However, with further temperature reduction, the time for crystallization increases. This occurs because the molecular movements required for crystal development become constrained kinetically by the lack of available thermal energy, as reflected by $t_{mr}$. The different time scales for crystallization via homogeneous and heterogeneous nucleation reflect the fact that catalyzed ice nucleation occurs at higher temperatures and requires fewer molecular rearrangements to occur. Fortunately, in practice solutes inhibit heterogeneous nucleation particularly well, tending to eliminate it at $C_v$. See text for more details. $T_g$, glass transition temperature; $T_h$, the homogeneous nucleation temperature; $t_{het}$, crystallization via heterogeneous nucleation; $t_{hom}$, crystallization via homogeneous nucleation;$T_m$, melting temperature. Adapted from Angell and Senapati [59].

## Kinetic aspects of ice avoidance

As described by Angell and Senapati, two time scales are relevant to crystal formation and growth (Figure 12.4) [59]. As the temperature of a solution drops below $T_m$, the time required for a given fraction of a solution to crystallize initially decreases (upper two curves). This is due to the increase in the driving force for crystallization as the temperature is reduced coupled with fast ice growth rates at relatively high sub-zero temperatures. On the contrary, as a solution cools, the time required for molecular rearrangements within a solution to occur (i.e. viscosity), also increases (lowest curve). The second time scale begins to dominate as the temperature is continuously lowered. Eventually, the time necessary for water molecules to arrange themselves into a crystalline structure becomes very long – longer than observable time scales, thus confining ice development to higher temperatures. Hence, at lower temperatures crystallization is thermodynamically favorable, but it does not occur on a normal time scale simply due to kinetic barriers that preclude the molecules from joining a crystal.

Boutron used a semi-empirical approach to describe the dependence of the total quantity of ice crystallized on the cooling rate for given solutions. Under the assumptions described in his work, and according to the most accurate model, the quantity of ice crystallized during cooling is given by equation 12.1:

$$-\ln(1-x^{\frac{1}{3}})+0.5\ln(1+x^{\frac{1}{3}}+1+x^{\frac{2}{3}}) + \sqrt{3}\,\mathrm{arctg}((\sqrt{3}x^{\frac{1}{3}})/(2+x^{\frac{1}{3}}))=\mathrm{k4}/|V| \quad [12.1]$$

where $x$ is the ratio of ice crystallized on cooling to the maximum crystallizable ice, $V$ is the cooling rate and k4 is an empirical constant [66]. In the same work, he also developed analytical expressions for devitrification on re-warming [66], and the predictive nature of these equations turned out to be quite accurate (see also [67, 68]). As an example based upon the figures published in Boutron's report, a binary solution consisting of 45% (w/w) ethylene glycol (EG) in water cooled at approximately 80°C/min results in approximately 1/3 of the amount of ice formed compared to the same solution cooled at ~20°C/min. Increasing the cooling rate to 160°C/min causes the total ice formation to decrease by ~95%.

In recent times, oocyte and sperm vitrification have focused on using the highest possible cooling rates and the minimum possible concentrations of cryoprotectant [6, 69, 70]. Although differential scanning calorimetry measurements in this cooling rate regime are not feasible, Toner *et al.* [71] were able to estimate the cooling rate necessary to achieve vitrification as a function of concentration even at very low concentrations by solving Boutron's equations. The results of these calculations, plus some experimental data for comparison [68], are provided in Figure 12.5 along with estimates for 0% solute [72, 73]. Figure 12.5 applies to solutions of pure cryoprotectants in water, but provides a reasonably good indication of the cooling rates needed to justify claims of vitrification using more dilute concentrations of cryoprotectant in carrier solutions when the effect of the latter as extra solute is taken into account.

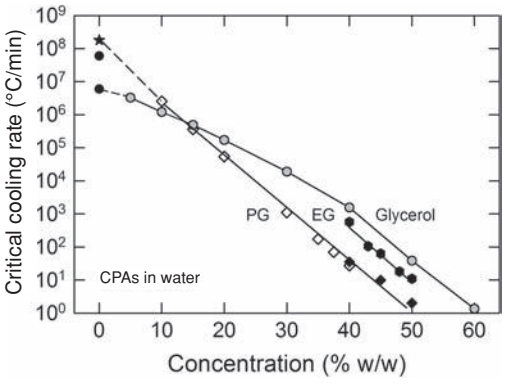


**Figure 12.5** Critical cooling rates for solutions of cryoprotectants in water in relation to their concentrations. Estimates of the $v_{ccr}$ for pure water from Bald [72] (star) and Bruggeller and Mayer [73] (circles) are shown at the upper left (0% solute). The gray circles for glycerol and open diamonds for propylene glycol (PG) are estimates (derived from [71] and used here with permission). (Ethylene glycol [EG] data and observed PG data (black diamonds) are from [68].) Modified from Fahy and Rall [3].

The warming rates necessary to avoid devitrification, i.e. the critical warming rates ($v_{cwr}$), are generally higher than those needed to vitrify (critical cooling rates, $v_{ccr}$) by several orders of magnitude. Baudot and Odagescu [68] reported, for example, the values of $v_{cwr}$ for solutions with 50, 45 and 40% (w/w) EG in water as 853, $1.04 \times 10^6$ and $1.08 \times 10^{10}$°C/min, respectively. Such rates reflect the increasingly short times needed for the growth of ice crystals to become detectable as homogeneous nucleation density becomes more and more astronomical. Fortunately, $v_{cwr}$ depends not only on the cryoprotectant concentration but also on the solute concentration of the carrier solution, which in some cases lowers $v_{cwr}$ as effectively as an equal mass of the cryoprotectant itself (Figure 12.6 [3]). Most vitrification solutions also contain sugars and other polymers; these compounds also significantly affect these critical rates [67, 75–77]. It is likely that intracellular proteins also affect the intracellular critical cooling and warming rates, but little information regarding the magnitude of the effect is available at this time.

The relationship between critical cooling rates and critical warming rates for several individual cryoprotectants is shown in Figure 12.7 [78–80], which highlights the challenge of avoiding devitrification after a vitreous state is achieved during cooling with lower concentrations of cryoprotectant. On the other hand, examples of the cooling and warming rates allowing survival of erythrocytes with two different cryoprotectants are also shown and indicate that survival is possible even when warming is orders of magnitude slower than the warming rate needed to prevent devitrification. Additional examples of this phenomenon have been tabulated elsewhere [81]. In further support, Seki and Mazur have recently determined the cooling and warming rates necessary for high survival

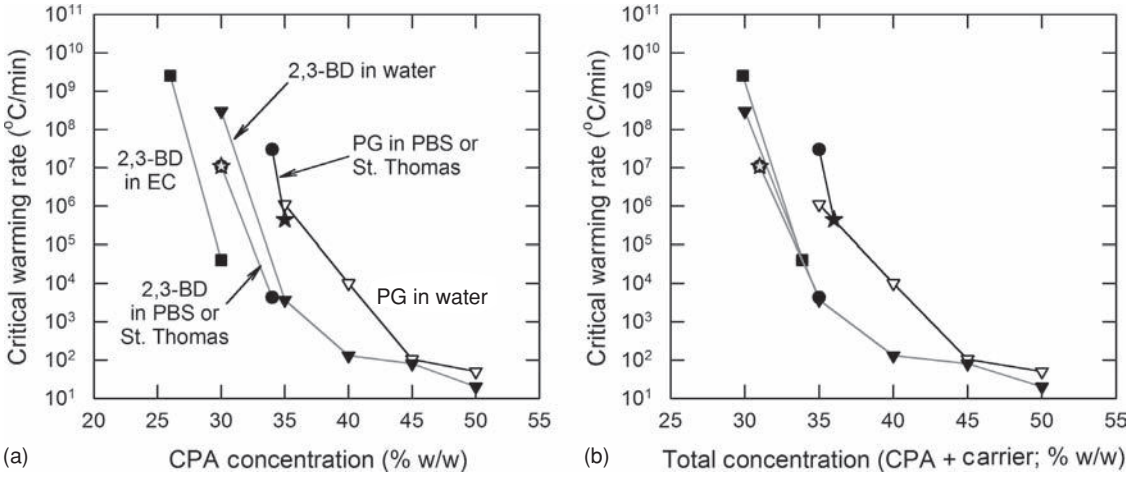


**Figure 12.6** Effect of the carrier (physiological support) solution on $v_{cwr}$. (a) When $v_{cwr}$ is plotted as a function of cryoprotective agent (CPA) concentration only, it can be seen that the presence of the sugar-rich Euro–Collins solution carrier (EC) can lower $v_{cwr}$ for 30% w/w 2,3-butanediol (2,3-BD) by ~4 orders of magnitude, whereas salt-rich carriers (PBS [circles] and St Thomas solution [stars]) have a smaller effect. (b) If the same data are plotted as a function of total solute concentration (CPA plus carrier solutes), it can be seen that the effect of the carrier is approximately the same, gram for gram, as that of the cryoprotectant, at least for 2,3-BD solutions. For solutions of propylene glycol (PG), the presence of salt carrier solutions may actually slightly increase $v_{cwr}$. These results allow the contribution of the carrier to vitrification solution stability to be estimated fairly easily as a rough rule of thumb. (Data from [66, 67, 74].) Modified from Fahy and Rall [3].

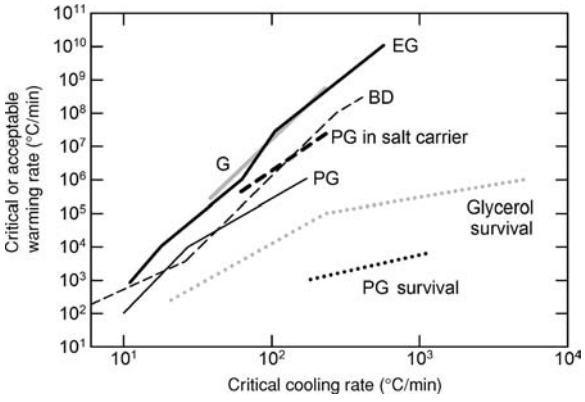


**Figure 12.7** Warming rates necessary for either survival (dotted lines) or the prevention of devitrification (solid or dashed lines) as a function of the critical cooling rate for solutions of cryoprotectants either in water or salt-based carrier solutions. Note that $v_{cwr}$ is two or more orders of magnitude greater than the $v_{ccr}$, a point often overlooked in the reproductive cryobiology literature. The values for survival are not meant to indicate the lowest warming rates compatible with survival, but are only known examples showing survival at far below the actual $v_{cwr}$ of similar solutions. (Physical data from [40, 67, 68, 78, 79]; survival data from [18, 41, 80].) BD, butanediol; EG, ethylene glycol; G, glycerol; PG, propylene glycol.

of mouse oocytes when using a more complex vitrification solution (EAFS 10/10, consisting of 10% [v/v] EG, 10.7% [v/v] acetamide, 24% [w/v] Ficoll-70 and 0.4 mol/l sucrose [in a stock solution of PB1 medium]) [82]. They found that maximum survival was attained with a cooling rate as low as 500°C/min provided the warming rate was approximately 3000°C/min (lower warming rates reduced survival). They report that the cooling rate needed to avoid ice formation in this solution is ≤500°C. Although we have not been able to find any reference to a critical warming rate for EAFS 10/10, it is likely to be >3000°C/min based on Figure 12.6.

Although Figure 12.7 supports the ability of cells to survive after devitrification, it and Seki and Mazur's observations, emphasize that attaining a high warming rate is more critical than attaining a high cooling rate, a point that is often ignored due to preoccupation only with ensuring vitrification during cooling. The same observations also make it apparent that survival alone is not unequivocal proof of vitrification [35]. This point is often overlooked in the reproductive cryobiology literature, and is particularly relevant to some of the current methodologies utilized for "vitrification." Many of these methodologies might be better characterized as nucleated vitrification procedures or as "one-way" vitrification methods in which appreciable freezing is avoided on the way down to below $T_g$, but not on the way back up to above $T_m$.

## Thermo-mechanical instability in vitreous materials and the problem of glass fracturing

Up to this point, we have discussed the stability of a vitreous solution only in terms of its propensity to crystallize. Thermo-mechanical instability can also develop during cooling, and is a concern for long-term storage of biomaterials at cryogenic temperatures [83], particularly for systems that are adversely affected by fracturing or deformation.

When forces are applied to a material, the material responds by changing shape; for example, if both ends of a wire are pulled in opposite directions (stress, $\sigma$), the wire will stretch (strain, $\varepsilon$). If the material returns to its initial size after the forces have been removed (think of stretching rubber), the material is said to have undergone elastic strain. Elastic strain is defined as the size of the change relative to the initial size; in the example of the rubber, the final length relative to the initial. Objects will also experience strain in the direction perpendicular to the applied stress (i.e. the rubber gets thinner as it is being stretched). The ratio of this transverse strain to the extension strain (in the direction of the applied force) is called the Poisson ratio. The proportionality between stress and strain for a given material is the Young's modulus (also referred to as the elastic modulus, $E = \sigma/\varepsilon$) and applies only to elastic strain. Viscous strain occurs when the stress exceeds a quantity known as the yield stress, and is associated with non-reversible rearrangement of molecular positions (think of taffy being pulled, which does not return to its original geometry). Viscous strain relieves at least some of the stress that builds up within a material.

Stress and strain issues in cryopreservation are usually associated with cooling and warming of the system. At high sub-zero temperatures, viscous strain occurs to a greater extent during cooling of a solution due to its relatively low viscosity. As the system continues to be cooled, the relative contribution of viscous strain decreases and elastic strain becomes more influential. Rabin *et al.* have defined the temperature at which the effects of these two strains are similar as the set temperature [84]. This temperature is usually near, but above, $T_g$ for solutions. Well above the set

temperature, elastic strain is insignificant and the solution acts like a liquid. Below the set temperature, the elastic strain dominates and the fluid acts like a solid (see Steif *et al.* [85] for more details).

To avoid stress buildup to the point of fracture, it is important to consider the effect of thermal gradients through the sample. During cooling at a constant rate, significant thermal gradients inevitably become established within all but very tiny systems. Above the set temperature, minimum stress exists within the system as cooling proceeds. As the system approaches the storage temperature (usually below the set temperature), the thermal gradients established during cooling can result in significant stress build-up within the system as the system approaches thermal equilibrium (e.g. as the still-pliant center of the system cools and pulls against the rigid outer layers). If the stress developed from this process exceeds the yield stress, fracturing results.

Using a continuum mechanics approach to modeling the stresses built up as a result of temperature gradients during cooling, Steif and colleagues developed an analytical expression to approximate the maximum tensile stress ($\sigma_{max}$, which occurs at the center of a sample) associated with various ideal geometries, given by the following equation:

$$\sigma_{max} = gE\beta\Delta T/(1 - v) \quad [12.2]$$

where E is the Young's modulus, $\beta$ is the coefficient of thermal expansion, $\Delta T$ is the temperature gradient during cooling, and v is the Poisson ratio of the system. The coefficient g depends upon the specific geometry of the sample, and is equal to 1/3, 1/2 and 2/5 for plate, cylindrical and spherical geometries, respectively. $\Delta T$ can be computed from the following equation:

$$\Delta T = f(Hd^2/\alpha) \quad [12.3]$$

with f being the coefficient for maximum temperature difference (equal to 1/8, 1/16 and 1/24 for plate, cylindrical, and spherical geometries, respectively), H the cooling rate, d the diameter of the sample, and $\alpha$ the thermal diffusivity [85].

Several important parameters at cryogenic temperatures, such as the viscosity and thermal diffusivity, are uncertain. This will influence the precision of such predictions. Nonetheless, such an analysis suggested that the strains that yield fracturing in vitrification solutions (0.23% for VS55 and 0.18% for DP6) are similar to those of brittle organic materials [84]. Further work along these lines may permit prediction of the exact conditions needed for fracture avoidance.

Fracturing is an even greater risk during warming than it is during cooling (e.g. [86]). It has also been demonstrated that fracturing is a concern with standard Cryo straws, and relatively slow initial warming of 1/4; cc straws after removal from $LN_2$ can eliminate fracture damage [87]. This latter observation supports the assertion that slow warming above the glass transition temperature allows the stress built up during cooling to be relieved as the viscosity decreases and the vitrified solution softens. This is the basis upon which a "5-second air-thaw" for a straw containing cryopreserved embryos prior to warming in a water bath was developed.

## Vitreous state storage below $T_g$

As mentioned above, the glass transition temperature of vitrification solutions is ~66–86°C above the boiling point of $LN_2$ (–196°C). Storage in $LN_2$ is common not because of the necessity of this temperature per se, but due to issues of convenience and stability. Many, perhaps most, of the current methods used to vitrify human oocytes use so-called open systems, where the sample comes in direct contact with $LN_2$. At this time, the issue of open systems, and the potential for contamination, is contentious [88, 89]. Storage in nitrogen vapor is seen by many as a superior alternative, and eventually may be the most common means to store vitrified samples.

An increasing amount of genetic resources is being banked at this time, and such an organized collection requires long-term stability for overall usefulness [90–92]. Clearly, one should choose a storage temperature that will match the requirements of storage time. The optimal storage temperature depends on the vitrification solution's $T_g$, nucleation characteristics, liability to damage from fracturing and on the biological system's viability as a function of time and temperature.

As described by Fahy and Rall, maintaining samples even above $T_g$ may still preserve viability for very long periods of time [3]. By combining the Vogel–Tammann–Fulcher (VTF) equation, which describes the temperature-dependence of viscosity, the Stokes–Einstein equation relating the diffusion coefficient to the viscosity and temperature and the relationship between diffusion distance, diffusion coefficient and time, one can calculate the time (t) necessary for the amount of diffusion to occur at a specific temperature

(T) to equal that obtained at time $t_1$ and a reference temperature ($T_1$):

$$t = t_1(T_1/T)\exp[B[(1/(T - T_0)) - 1/(T_1 - T_0)]],$$

where $T_0$ estimates the limiting temperature for structural change [3]. Figure 12.8a provides an example of the use of curve-fitting of the VTF equation for a specific vitrification solution (M22) [58], and Figure 12.8b shows how the results can be applied to the estimation of acceptable storage times. This particular calculation is based upon the assumption that biological damage due to the vitrification solution is dependent on diffusion, and that the total accumulated damage is equivalent to damage caused by exposure to a vitrification solution for 10 s at 0°C. As can be seen, even at temperatures well above $T_g$, safe storage times are predicted to be quite long. For storage below $T_g$, damage resulting from diffusional processes is likely to be minuscule.

Such calculations do not, however, take into account the fact that cubic ice nucleation (which evidently requires minimal diffusion) may still occur at temperatures even below $T_g$ (see Figure 12.2). Thus, empirical research results that define a critical warming rate for a system when storage near $T_g$ is very brief may not extrapolate when storage times are extended, for devitrification may be a greater concern in the later case [93]. On the other hand, it is known that jugular veins stored at –130°C (just 7°C below $T_g$) for 4 months show no increase in damage compared to storage for only 24 h (both showed 80% recovery versus controls, [94]). Whether chilling-sensitive systems will show a similar degree of stability remains to be seen.

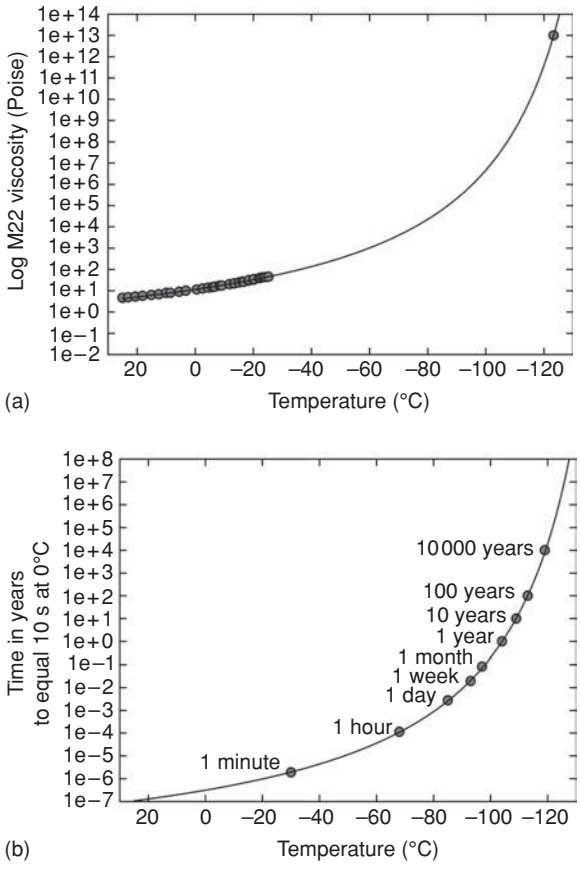


**Figure 12.8** In (a), the fit of the Vogel–Tammann–Fulcher (VTF) equation to viscosity data for M22 is shown. The point at the upper right is an estimate of the viscosity at the glass transition temperature ($10^{13}$ Poise). The VTF equation is $\eta(T) = A\exp[B/(T - T_0)]$ where $\eta$ is the solution viscosity, T is temperature and $T_0$ is the estimated limiting temperature for structural change, which approximates the Kauzmann temperature (see [3, 62] for more details). For this example, A = 0.009, B = 1112, $T_0$ = –155.4°C and T is in °C. (The composition of M22 can be found in Table 2 of [58].) Part (b) combines the values of B and $T_0$ from (a) with the Stokes–Einstein equation [which relates the diffusion coefficient for a substance (D) to the temperature-dependent viscosity $\eta(T)$: $D = K_B T / (6\pi \eta(T r)$, where $K_B$ is the Boltzmann constant, T the absolute temperature and r the particle radius], and the equation for the time required for a given amount of diffusion to take place at a particular temperature [3], to provide an estimate by means of Equation 12.4 of the storage times for viability maintenance as a function of the storage temperature. Even at temperatures nearly 80°C above the temperature of liquid nitrogen (–196°C), it is estimated that storage times exceed several centuries. See text for more details.

## Vitrification solutions and vitrification solution toxicity

The composition of vitrification solutions is an essential consideration given the relatively high concentrations of cryoprotectants required. Many mixtures of cryoprotective agents have been tested as vitrification solutions (VSs) for reproductive cells, tissues, and organs over the past two decades [95, 96]. Ali and Shelton examined a large number of mixtures in search of the most useful combinations for mouse morulae [97, 98], and have recently published a useful summary of their searches and conclusions [95]. Other investigators have only compared relatively small numbers of solutions for vitrification purposes for reproductive cells [99–101]. A recent meta-analysis of the published literature of full reports over the past 10 years identified fewer than 10 vitrification solutions having been tested on in vivo matured human oocytes to date [102]. This is undoubtedly due, at least in part, to the

difficulty of using healthy mature human oocytes for experimental purposes.

After years of experience, the choice of compositions utilized by many investigators still seems nearly random, and little consensus seems to exist as to which solutions are optimal for which reproductive cells. The empirical success of the Kuwayama method for oocytes [103] seems to be encouraging more use of 15% $Me_2SO$ + 15% EG as the basic permeating component of the VS for human oocytes [2, 104, 105], but it has not been shown that this solution is actually better than competing formulas when used in the same protocol [102].

The wide range of empirical solutions in the literature is not surprising. Analytically, the problem of mapping the full range of available composition–temperature–time design space is enormous. How many agents will be used? In what proportions? At what total concentration? With what balance of permeating and non-permeating agents, as chosen on the basis of what rationale? With what loading and unloading protocol, developed on what basis, and with what accompanying temperatures? Dissolved in what carrier solution and combined with that solution in what way? Compared to what controls? And all justified on the basis of what functional endpoints with what cells? Given the large number of choices available, the differing intuitions of different investigators and the limited inclination and/or ability of most laboratories to map out compositional and procedural variables in detail, a broad spectrum of proposed solutions is only to be expected.

However, many guiding principles have been developed that may help to provide direction to future studies and that may eventually lead to more of a consensus concerning how to design, introduce and remove VSs. We therefore briefly re-examine here the principles of good VS construction and use as we presently understand them.

## Good VSs require bad glass-forming agents

According to an analysis published in 2004 [65], the compositional variable upon which toxicity depends is q, which is defined as $M_W/M_{PG}$, in which $M_W$ is the molarity of water in the solution and $M_{PG}$ is the molarity of water-bonding groups (polar groups) on the permeating cryoprotectants of the VS. Good glass formers vitrify water at low glass former concentrations and therefore $M_W$ and q are relatively high at the threshold concentration needed for the solution to vitrify under standard conditions (qv*, pronounced "cue vee star"). According to analyses of many VSs tested on rabbit renal cortical slices, viability is high when qv* is low, and vice versa, indicating that weak glass forming agents are less toxic than strong ones. A similar trend is apparent from the results of Ali and Shelton [98] when the toxicity of 13 vitrification solutions on mouse morulae is assessed [S. F. Mullen, unpublished results].

This is interpreted to mean that it is preferable to choose cryoprotectants that compete less strongly with cellular constituents for access to water, and that water is actually more available to hydrate biomolecules in VSs that have lower absolute water concentrations at qv* [65]. However, it is often preferable to use mixtures of cryoprotectants rather than attempting to select only the weakest available glass former because the concentrations needed for any one agent to vitrify tend to be high enough to introduce specific toxic effects for that agent that defeat its theoretical advantages [65]. Nevertheless, Rall's choice of glycerol + albumin (VS3 [106]) is consistent with qv* theory because glycerol is a particularly ineffective glass former, polar group for polar group, compared to other agents. Unfortunately, glycerol tends to be limited by its low permeability, and in some systems is able to dramatically lower adenosine triphosphate (ATP) levels by being phosphorylated at the expense of ATP [107].

MacFarlane and colleagues have examined the physicochemical basis of glass formation in aqueous cryoprotectant solutions by nuclear magnetic resonance (NMR) spectroscopy techniques, and were able to establish that glass-forming efficacy is directly related to the water-cryoprotectant hydrogen bond strength (cryoprotectant basicity) [26, 108]. These fundamental observations might in principle be used to quantitatively relate qv* to hydrogen bond strength and therefore to relate viability directly to hydrogen bond strength, but this depth of analysis has not to date been pursued.

## Consider employing cryoprotectant toxicity neutralization

There is one exception to the rule that weak glass-forming agents should be used, and that arises from the phenomenon of cryoprotectant toxicity neutralization (CTN), which has recently been reviewed in detail [109]. Certain amides have the remarkable property

of having toxicity that can be blocked by the simultaneous presence of $Me_2SO$, such that, for example, a 50% w/v total concentration resulting from the combination of 20% w/v formamide with 30% w/v $Me_2SO$ can have no toxicity even though 20% w/v formamide by itself can lower viability by 60% [110]. The mechanisms involved are unknown, but sufficient clues are available to enable mechanisms to be elucidated [109]. In the meantime, CTN enables the exceptionally poor vitrification tendency of amides to be traded off against the strong glass-forming tendency of $Me_2SO$ and supplemented with the use of other intrinsically weak glass formers to enable solutions of exceptionally low toxicity, high concentration and high overall stability against ice formation [58, 65, 111].

The use of amides in VSs can be controversial when considered outside the context of CTN. However, the toxicological effects of amides in vivo are not meaningful in the context of low temperature addition and removal in the presence of $Me_2SO$ in vitro, and can be avoided if necessary by ensuring that all amides are removed prior to re-warming to 37°C or by very rapid washout at 37°C.

The choice of acetamide in the original VS1 [55] solution may have been suboptimal [65, 109]. Formamide currently appears to be the amide of choice, combining high permeability [112], exceptionally poor glass-forming ability [24], lack of denaturing character [109], full toxicity neutralization potential [109] and lack of any documented mutagenicity or carcinogenicity. Thus far, CTN has been demonstrated only in kidney and liver tissue, and seems not to apply to brain tissue. Nevertheless, solutions based on CTN have been effective for brain slices [113], mouse oocytes [65] and many other systems.

## Include extracellular agents in moderation

The vitrification tendency of cytoplasm and of organelles will differ from that of the extracellular medium due to the presence of intracellular proteins and membranes. It was shown in 1984 that this enables some reduction in the level of permeating cryoprotectant to be accomplished, intracellular protein being balanced by extracellular polymers [22], and it is now known that total solution toxicity depends more on permeating cryoprotectants than on impermeants [58, 65, 109]. What is less clear is the limit beyond which this principle cannot or should not be extended. Excessive reliance on impermeants to achieve vitrification may in principle reduce cell viability through excessive cell shrinkage [114, 115], exacerbating chilling injury as a result of too much cell shrinkage [58] and allowing IIF if the glass-forming tendency or anti-nucleating effectiveness of extracellular polymers such as ice blockers significantly exceeds that of intracellular proteins. Cell shrinkage secondary to the use of polymers may exacerbate hypertonic osmotic injury upon cryoprotectant addition but will also limit the amount of cryoprotectant that enters cells and, consequently, will simplify the removal of that cryoprotectant after storage [22].

## Consider special additives

In recent years, special additives, including most prominently the ice blockers X-1000 [116] and Z-1000 [117] but also including ice-growth inhibitors [111; 21st Century Medicine, unpublished results], 3-methoxy-1,2-propanediol [58, 118, 119] and, if needed, N-methylformamide [58], have been introduced to eliminate heterogeneous nucleation and enhance extracellular and intracellular vitrification without increasing toxicity when used in low concentrations. These additives and solutions based on them are commercially available and have been efficacious for numerous diverse systems outside the realm of reproductive cryobiology, and have allowed good recovery of vitrified mouse oocytes [65].

## Choose your carrier solution carefully

The toxicity and glass-forming ability of a vitrification solution depend to a significant extent on the carrier or physiological support solution in which the cryoprotective agents of the vitrification solution are dissolved (see Figure 12.6; [120]). At 21st Century Medicine, we use an LM5 carrier solution, which enhances the activity of ice-blocking agents and sustains cell viability well in the presence of high cryoprotectant concentrations [58, 65, 121].

## Define your needs

The toxicity and ice prevention efficacy of a VS both increase as the total solution concentration increases. The former effect should be minimized by determining the minimum required size of the latter effect and limiting total concentration to what is actually needed. This point is emphasized particularly because vitrification tendency is sometimes assessed in unnecessarily large increments of total solution concentration, such

as increments of 5% or 0.5 mol/l [95], when smaller increments might both suffice for attaining sufficient solution stability and result in important reductions in overall toxicity.

## Eliminate osmotic effects

The negative effects of VSs can arise, of course, not only from true biochemical toxicity but also from osmotic effects if the latter are not carefully excluded. Although mass transfer modeling is the best way to estimate appropriate protocols for adding and removing VSs while avoiding osmotic injury [122–124], empirical testing is also required and may be sufficient if the investigator is sufficiently thorough in verifying that further increases in exposure time at a given concentration, further reductions in concentration step size or further decreases in the rate of change of concentration do not result in additional protection [125]. In general, exponential rates of increase and decrease in concentration will minimize both toxicity and osmotic effects [109, 126].

Osmotic effects will be maximized at lower temperatures, but lower temperatures are generally necessary to minimize toxicity unless chilling injury near 0°C is so severe as to preclude lower temperature cryoprotectant addition [6, 127]. For systems in which chilling injury is not exacerbated by excessive cell shrinkage, low temperature addition of the final VS may only need to allow cytoplasmic concentration by the exosmosis of water, cryoprotectant uptake by cells being unnecessary [22].

## Summary

Vitrification is becoming an increasingly common means to cryopreserve reproductive cells and tissues. Efforts to understand the physics of vitrification have been underway for decades, and the basic principles of vitrification are now reasonably well understood, although many important practical details remain to be investigated. Achieving vitrification in a laboratory setting is generally believed to require the use of multimolar concentrations of cryoprotectants, which have the potential to be toxic to living cells. Fortunately, some general principles underlying the relationship between molecular structure, vitrifiability, and toxicity have been elucidated in recent years, increasing our ability to develop vitrification solutions on a rational basis. While the avoidance of the damaging effects of ice formation is the basis for choosing vitrification as a means of cryopreservation, mechanical damage due to fracturing of the vitreous material can also cause serious injury, in some cases negating the benefits of vitrification. However, utilizing interdisciplinary knowledge of the fundamental principles of vitrification, the fundamental cryobiology of the cells being preserved and the derived parameters associated with current technology, continued improvements in vitrification methods for reproductive cells, tissues and organs should be possible, enabling the achievement of superior fertility preservation results.

## Acknowledgements

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Chapter 13

# Hormonal suppression for fertility preservation in the male

Gunapala Shetty

## Introduction

Radiation and chemotherapeutic regimens required for life-threatening diseases, such as cancer or exposure to environmental toxicants, may jeopardize the fertility of men of reproductive age causing permanent or temporary azoospermia. Young pre-pubertal patients who cannot supply a semen sample for cryopreservation of sperm have particularly poor fertility prognosis. In the USA alone about 17 000 men aged 15–45 years old are diagnosed each year with Hodgkin's disease, lymphoma, bone and soft tissue sarcomas, testicular cancer or leukemia. Of these, over 3000 are treated with doses of alkylating agents, platinum drugs or radiation that are sufficient to induce prolonged azoospermia. In addition, over 6000 boys under the age of 15 are diagnosed each year with cancer, including leukemia, nervous system tumors, lymphomas and other solid tumors. About 80% of them receive chemotherapy or gonadal radiation, and about 550 of the long-term survivors are azoospermic when they reach adulthood.

Environmental and occupational toxicants can also produce prolonged azoospermia. This was most dramatically shown with dibromochloropropane (DBCP), as highly exposed manufacturing and agricultural workers had an increased incidence of azoospermia [1, 2]. In addition, sterility can be developed during development such as due to cryptorchidism or aging.

Methods to prevent these effects on male fertility and to restore normal testicular function are of great importance. A variety of biochemical and biological approaches (thiol radioprotectors, prostaglandin analogues, growth factors, blockers of apoptotic pathways, and reduction in blood flow) have been tested to protect the testes in experimental animal model systems against radiation and chemotherapy (reviewed in [3]). However, utmost research interest in this field, including all clinical trials, has involved hormonal modulation in attempts to prevent or reverse damage to the germ line from radiotherapy and chemotherapy. In this chapter, I will discuss the current knowledge of hormonal suppression as a means to preserve or restore fertility in males.

## History and hypotheses

The use of hormone suppression for protecting gonadal function after cytotoxic exposure is based on the observation that non-cycling cells are generally more resistant to killing by certain toxicants, particularly anti-neoplastic agents, than are rapidly proliferating cells.

The mechanism originally proposed for the protection of spermatogenesis was that the interruption of the pituitary–gonadal axis would reduce the rate of spermatogenesis and render the resting testis more resistant to the effects of chemotherapy [4]. This hypothesis was based on the claim that pre-treatment with gonadotropin-releasing hormone (GnRH) analogue – which in rodents produces reversible inhibition of follicle stimulating hormone (FSH) and luteinizing hormone (LH) secretion and severe reduction in serum testosterone levels – protected spermatogenesis in the mouse from cyclophosphamide-induced damage. However, attempts to repeat these original observations using more quantitative endpoints revealed that there was no protection [5]. Since suppression of gonadotropins and testosterone only

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blocks the completion of spermatogenesis but has no effect on the kinetics of the early spermatogenic cells [6], the premise on which the proposed mechanism was based was incorrect and a negative outcome should be expected. Furthermore, the stem spermatogonia, which are more important targets than the differentiating germ cells for the long-term effects of cytotoxic damage, did not appear to be affected at all by hormonal suppression.

Despite the failure until now to observe protective effects in mice, it has been convincingly shown that suppression of gonadotropins and intratesticular testosterone levels prior to, during or after exposure of rats to chemotherapy, radiation or other cytotoxic agents enhances the subsequent recovery of spermatogenesis, as discussed below. Thus other mechanisms must be involved and careful attention must be given to the species used.

## Effect of cytotoxic agents on testicular function

The testis consists of the seminiferous (or germinal) epithelium arranged in tubules and endocrine components (testosterone-producing Leydig cells) in the interstitial region between the tubules. The seminiferous tubules contain the germ cells, which consist of stem and differentiating spermatogonia, spermatocytes, spermatids and sperm and the Sertoli cells, which support and regulate germ cell differentiation. Among the germ cells, the differentiating spermatogonia proliferate most actively and are extremely susceptible to cytotoxic agents. In contrast, the Leydig and Sertoli cells, which do not proliferate in adults, survive most cytotoxic therapies. These cells may, however, suffer functional damage. Frequently, following cytotoxic therapies, germ cells appear to be absent, and the tubules contain only Sertoli cells. This could be a result of the killing of the spermatogenic stem cells, the loss of the ability of the somatic cells to support the differentiation of a few surviving stem cells or a combination of the two.

The eventual recovery of sperm production depends on the survival of the spermatogonial stem cells and their ability to differentiate after exposure to cytotoxic agents. The reduction should be temporary provided the stem spermatogonia survive. However, stem spermatogonia are killed by some of these agents at varying degrees and recover only gradually, resulting in prolonged reductions of sperm count; in the mouse this reduction is directly related to stem cell death [7]. It is rare that surviving stem spermatogonia fail to differentiate in mouse testes [8]. In contrast, after exposure of rats to several anti-neoplastic agents [9] and other toxicants [10], the stem spermatogonia that survive are blocked from differentiating and their progeny undergo apoptosis instead [11]. This block has been shown to be a result of damage to the somatic environment within the testis, not to the spermatogonia [12]. There is, however, no evidence of a similar spermatogonial block in monkeys [13]. In men, surviving stem cells can remain in the testis but fail to differentiate into sperm for several years after cytotoxic insult, so delayed recovery is possible [14]. At lower doses of these agents, recovery to normospermic levels can occur within 1–3 years, but at higher doses, azoospermia can be more prolonged or even permanent.

The loss of germ cells has secondary effects on the hypothalamic–pituitary–gonadal axis. Inhibin secretion by the Sertoli cells declines and, consequently, serum FSH levels rise. Testicular blood flow is reduced, resulting in less testosterone being distributed into the circulation [15]. Therefore LH levels increase to maintain constant serum testosterone levels. The reduction in the testis size and increased LH levels also contribute to an increased concentration of testosterone within the testis.

## Hormone suppression studies in animal models

Several studies support the conclusion that gonadotropin suppression does not protect spermatogenesis in mice from damage [8, 16, 17]. In contrast, numerous reports suggest that hormone suppression protects rat testes from damage due to irradiation, procarbazine, doxorubicin, an indenopyridine compound and heating [18–26] and enhances future fertility in the face of these toxicants. In addition to direct suppression of gonadotropins with GnRH agonists and antagonists, which also results in both direct and secondary suppression of intratesticular testosterone, these studies also utilized combinations of GnRH analogues with antiandrogens, systemic physiological doses of testosterone (which suppresses gonadotropins and results in reduced intratesticular testosterone levels), progestins (which are very effective at suppressing gonadotropins but have weak androgenic activity) and estrogens (which

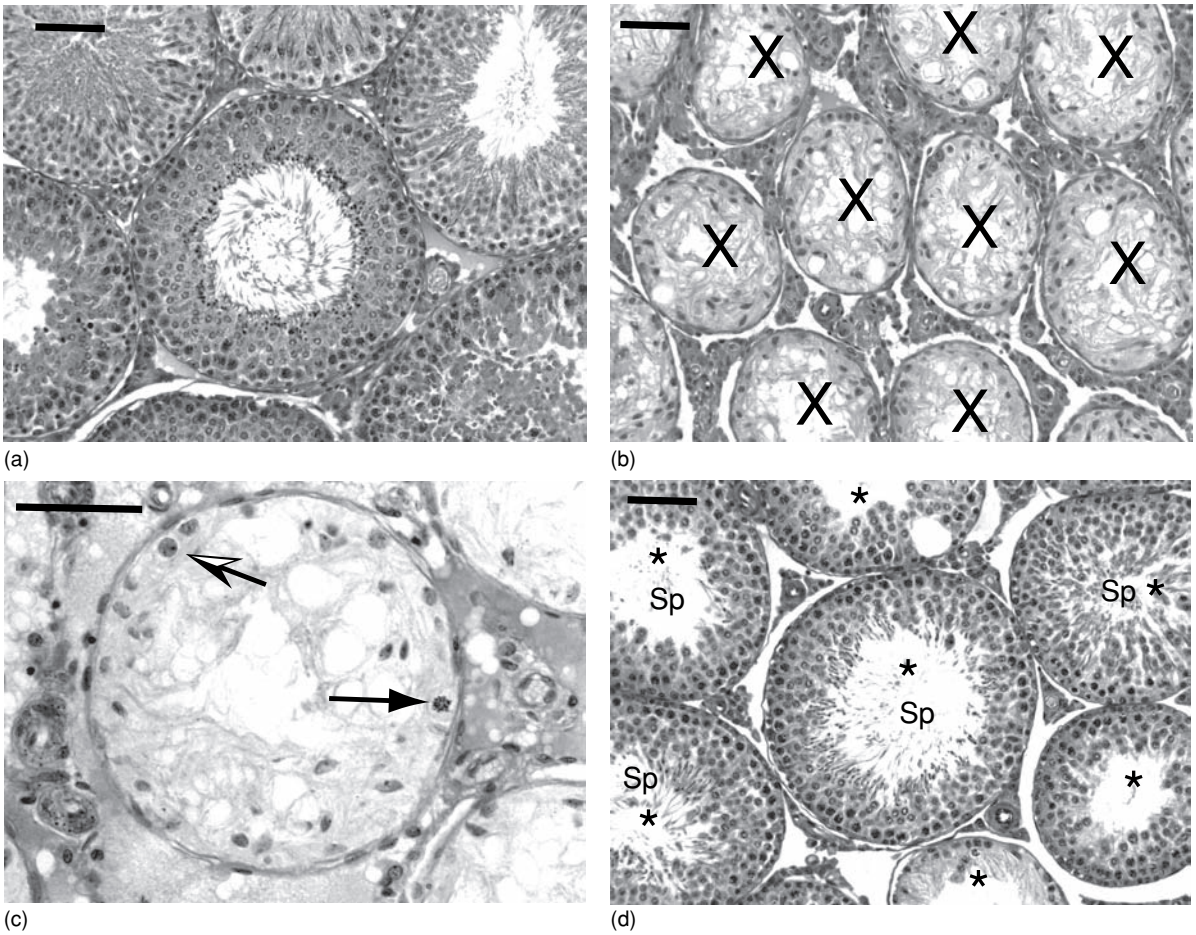


**Figure 13.1** Photomicrographs of Lewis brown–Norway $F_1$ hybrid (LBNF1) rat testes sections showing normal spermatogenesis in control testis (a), radiation-induced block in the differentiation of spermatogonia (b,c) and the reversal of this block by gonadotropin-releasing hormone (GnRH) antagonist treatment. The testes were harvested 13 weeks after 5 Gy irradiation with or without GnRH antagonist treatment during weeks 3–7 after irradiation. Note that with no GnRH antagonist treatment all tubules are atrophic (X) and contain only Sertoli cells and type A spermatogonia (b), with normal (half-open arrow) and dividing (filled arrow) spermatogonia shown at higher magnification (c). With GnRH antagonist treatment all tubules are repopulating (*) with mature spermatids in many of them (Sp) (d). Bars = 50 μm.

both suppress gonadotropins and inhibit testosterone synthesis). It should be noted that in all these studies protection was not assessed directly at the time of cytotoxic exposure, but rather by the enhanced ability of spermatogenesis to recover from surviving stem cells [27], which is actually the most relevant endpoint for future fertility.

Attempts to protect spermatogenesis in other animal species (dog, monkey) have not yielded any reproducibly positive results. Although one group reported that GnRH agonist shortened the time for recovery of spermatogenesis after treatment of dogs with cyclophosphamide, cisplatin or radiation [28], another study reported potentiation of the damage [29]. Studies using hormone suppression in monkeys have not convincingly demonstrated enhanced recovery of spermatogenesis following gonadotoxic injury. Although one preliminary report based on a total of only three baboons suggested that hormone suppression might decrease the gonadal damage from cyclophosphamide [30], two larger studies using an adequate number of macaques showed neither protection [31] nor stimulation [13] of recovery of spermatogenesis from radiation damage by hormone suppression treatment.

We proposed that cytotoxic exposure of normal testis (Figure 13.1a) produces a pronounced block in the differentiation of surviving stem spermatogonia

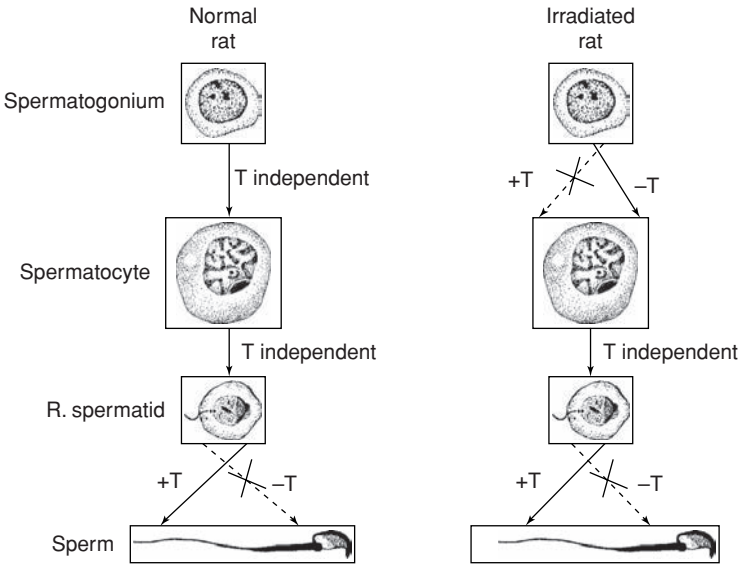


**Figure 13.2.** Diagrammatic representation of the role of testosterone in regulating spermatogenesis at various steps during normal spermatogenesis and in the irradiated rat testes. In normal rats, spermatogonial differentiation is qualitatively independent of testosterone, with the absolute requirement of testosterone during spermiogenesis. However, after irradiation testosterone inhibits the differentiation of spermatogonia.

in rat testes (Figure 13.1b,c) and prevention of this block is the mechanism by which hormone suppression appears to protect spermatogenesis from toxicant exposure [27]. It is important to note that many of the studies showing protection involved subchronic exposure to the cytotoxic agent, so the hormonal suppression was given after the initial exposures, and in some cases even extended beyond the last exposure [32]. Furthermore, when the hormonal suppression was administered to the rats only after the cytotoxic insult, either immediately or after a delay of several months, the numbers of differentiated germ cells still dramatically increased [33]. However, because testosterone, which is required for spermatid differentiation, was suppressed, spermatogenesis proceeded only to the round spermatid stage: no sperm were produced. Nevertheless, when additional time without further suppressive treatment was allowed before the rats were euthanized, all tubules showed almost complete spermatogenic recovery (Figure 13.1d), sperm counts increased and the fertility of the rats significantly increased [27]. This phenomenon appears to be quite general: post-treatment with GnRH agonists or antagonists, with or without antiandrogen, low dose systemic testosterone, estradiol or hypophysectomy are all effective at stimulating recovery [34–36], and recovery has been stimulated following gonadal toxicity from radiation, procarbazine [37], busulfan [38], hexanedione [39], dibromochloropropane [40], an indenopyridine compound [25] or heat treatment [41]. The endogenous hormone primarily responsible for the inhibition of spermatogonial differentiation in toxicant-treated rats was testosterone, although FSH also had a minor inhibitory effect [35], and other exogenously administered androgens were also inhibitory [34]. It was also observed that hormonal suppression after irradiation of mouse testes modestly but significantly increased the percentage of tubules in which differentiation of surviving spermatogonia occurred [G. Wang and M. L. Meistrich, personal communication].

Hormonal suppression with GnRH analogues or hypophysectomy has also been shown to promote the survival and differentiation of spermatogonia that are transplanted into testes of animals that were depleted of endogenous stem cells. Although the effects were most dramatic when rat testes depleted by cytotoxic treatments were used as recipients [12, 42], enhanced proliferation and differentiation of transplanted spermatogonia were also observed when mouse testes were used as recipients [43–45; G. Wang and M. L. Meistrich, personal communication].

The inhibition of spermatogenesis by testosterone after cytotoxic exposure such as radiation appears to be contradictory to its well-studied role in stimulating and maintaining spermatogenesis (Figure 13.2). In normal unirradiated and irradiated rats, testosterone has a similar role in spermiogenesis, as shown by the failure to find cells that have developed past the round spermatid stage in both these cases when testosterone

**Table 13.1** Results of hormone suppression treatments given before and during cytotoxic therapy on spermatogenic recovery in men

| Disease | Cytotoxic therapy[a] | Hormone treatment | Recovery[b] | | References |
|---|---|---|---|---|---|
| | | | Hormone-treated | Controls | |
| Hodgkin's | MOPP 3–6 cycles | GnRH agonist | 1/5 | No controls | Johnson *et al.* [48] |
| Hodgkin's | MVPP, ChlVPP | GnRH agonist + testosterone | 0/20 | 0/10 | Waxman *et al.* [49] |
| Hodgkin's | MOPP ~4 cycles | Testosterone | ≈ 70%/23[c] | ≈ 70%/22[c] | Redman and Bajorunas [50] |
| Testis Ca | PVB + ADR/CY + radiation | Medroxy-progesterone | 0/4 (2/12)[d] | 2/3 (7/13)[d] | Fossa *et al.* [51] |
| Testis Ca | PVB | GnRH agonist | 6/6 | 8/8 | Kreuser *et al.* [52] |
| Testis Ca (Seminoma) | Radiation | GnRH agonist + antiandrogen | 12/12 | 8/8 | Brennemann *et al.* [53] |
| Nephritis | Cyclophos-phamide | Testosterone | 5/5 | 1/5 | Masala *et al.* [54] |

Ca, cancer; GnRH, gonadotropin-releasing hormone.
[a] Chemotherapy regimens are as follows: ADR/CY, adriamycin, cyclophosphamide; ChlVPP, chlorambucil, vinblastine, procarbazine and prednisone; MOPP, mechlorethamine, vincristine, procarbazine and prednisone; MVPP, mechlorethamine, vinblastine, procarbazine and prednisone; PVB, cisplatin, vinblastine, bleomycin.
[b] Fraction of men recovering testicular function as assessed by restoration of sperm counts to normospermic levels unless otherwise noted.
[c] Actuarial recovery calculated by Kaplan–Meier analysis.
[d] Recovery assessed by restoration of follicle stimulating hormone (FSH) levels to within the normal control range.

is suppressed. But, unlike normal rats in which spermatogonial survival and differentiation are qualitatively independent of testosterone, in irradiated rats the survival and differentiation of type A spermatogonia are inhibited by this hormone.

The mechanism by which somatic cells in rat testis are protected from damage if testosterone suppression treatment is given at the time of cytotoxic exposure is not known. Neither is the mechanism by which testosterone suppression after cytotoxic exposure enhances the ability of the somatic elements of the testis to maintain the differentiation of spermatogonia. Previous studies ruled out the possibility that the protective effect of hormonal suppression given before cytotoxic drug exposure was due to reduced delivery to the tissue or altered metabolism of the drug [46]. We have recently shown that the block in spermatogonial differentiation in rats treated with cytotoxic agents is associated with the increased levels of interstitial edema in the testes [47]. Based on data indicating that more rapid stimulation of recovery of spermatogonial differentiation in irradiated rats was achieved by elimination of Leydig cells with ethane dimethane sulfonate than with total androgen ablation [G. Shetty and M. L. Meistrich, unpublished data], we are analyzing the role of Leydig cells as targets for the testosterone-induced inhibition of spermatogonial differentiation in toxicant-treated rats. Recent studies in rats also suggested a direct stimulation of spermatogonial differentiation by estradiol after irradiation, in addition to its indirect effect through suppression of testosterone [36]. It is yet to be determined whether the spermatogonia having estrogen receptor beta (ER-β) receptors are the target for estradiol, unlike androgens, which have receptors only on somatic cells.

## Clinical trials

Seven clinical trials have been performed in attempts to demonstrate protection of spermatogenesis in humans by hormone suppression treatment before and during cytotoxic therapy, but six indicated no protection (Table 13.1 [48–54]). Three of the trials involved patients treated for Hodgkin's lymphoma and three involved testicular cancer patients. Treatment with GnRH agonist resulted in only 20% of

patients recovering sperm count after cessation of chemotherapy [48]. However, no concurrent control group of patients receiving similar regimens of chemotherapy without GnRH agonist were enrolled in this study. In another study, hormone suppression with testosterone combined with GnRH agonist prior to and during chemotherapy was randomized with no hormonal suppression [49], but none of the patients from the control and treated groups showed evidence of recovery of spermatogenesis at 1–3 years after completion of therapy. Suppression of gonadotropins and intratesticular testosterone levels with testosterone injections alone during treatment [50] also did not provide gonadal protection benefit: 70% of the patients in both the treated and control group showed spermatogenic recovery at 3 years. Suppression of gonadotropins with medroxyprogesterone acetate during chemotherapy combined with radiotherapy did not improve the recovery of sperm count or normalize FSH levels, which was used as a surrogate for sperm count in patients in whom sperm counts were unavailable; indeed, they appeared to be lower in the patients receiving concurrent treatment with hormonal suppression than in controls [51]. Two more studies used GnRH agonist [52] or GnRH agonist plus an antiandrogen (cyproterone acetate) [53] prior to and for the duration of chemotherapy or radiation therapy, respectively. In these studies the chemotherapeutic regimen was only 2 courses of cisplatin, vinblastin and bleomycin (PVB) and the gonadal dose of radiation was 0.2 Gy, which allowed spontaneous recovery of sperm counts in all the control patients within 2 years. The time course of recovery of spermatogenesis after chemotherapy was identical for the groups of patients with or without GnRH-agonist treatment. Although fluctuations in sperm counts made it difficult to determine whether the time course of recovery of spermatogenesis was affected by hormonal treatment, the time course of reduction of elevated FSH levels back to pre-treatment values was similar in controls and in the patient groups treated with GnRH agonist and antiandrogen.

The one study that demonstrated hormonal treatment preservation of sperm production in men involved testosterone therapy of men who received cyclophosphamide as an immunosuppressive therapy for nephrotic syndrome [54]. During the treatment, the testosterone suppressed gonadotropin levels and suppressed the completion of spermatogenesis. All but one of the men who received cyclophosphamide alone remained azoospermic 6 months after the end of immunosuppressive therapy, whereas sperm concentrations returned to normal in all 5 men who received cyclophosphamide in combination with testosterone therapy.

The one attempt to restore spermatogenesis by steroid hormone suppression after cytotoxic therapy was also unsuccessful [55]. Seven men with azoospermia secondary to high dose chemotherapy and/or radiation therapy for leukemia or lymphoma in childhood were treated with medroxyprogesterone acetate combined with testosterone to suppress gonadotropin and likely intratesticular testosterone levels many years after the anti-cancer treatment. None of the men recovered any sperm production during the 24-week follow-up after the end of hormonal treatment.

Even if the hormonal suppressive treatments that were successful in protecting and stimulating spermatogenic recovery in rats are applicable to human males, there may be many reasons for the unsuccessful outcomes of the aforementioned clinical trials. The use of testosterone or medroxyprogesterone either alone [50, 51, 55] or combined with a GnRH analogue [49] is suboptimal given that, in animal studies, both of these steroids inhibit the effects of GnRH analogues in stimulating recovery of spermatogenesis after cytotoxic damage [34, 56]. The number of patients and controls studied was small [48] and the cancer therapies variable. Some treatment regimens were not sufficiently gonadotoxic to cause sterility [52, 53]; conversely some regimens may have delivered doses well above that needed to ablate all spermatogonial stem cells, since no evidence of spermatogenesis was observed in almost all patients even after many years [48, 49, 55]. Thus, the application of these procedures to humans remains uncertain.

## Analysis of interspecies differences

The results of studies of protection of long-term gonadal function by hormonal suppression in experimental animals and humans are summarized in Table 13.2 [5, 8, 13, 16–21, 31, 33, 37, 38, 40, 48–55, 57–59]. The dramatic stimulation of recovery of spermatogenesis by hormone suppression in toxicant-treated rats is in contrast to the less marked effects observed in mice, the absence of stimulation in macaques, and generally negative but variable results in human.

**Table 13.2** Summary of effects of hormone suppression on protection and/or stimulation of recovery of spermatogenesis after cytotoxic treatment in different species

| | | Effect of timing of hormone suppression relative to cytotoxic treatment | | | |
|---|---|---|---|---|---|
| **Species** | **Treatment** | **Before** | **Immediately after** | **After a delay** | **References** |
| Mouse | Procarbazine, doxorubicin, cyclophosphamide | – | | n.d. | Da Cunha *et al.* [5]; Crawford *et al.* [17] |
| | Cisplatin | – | n.d. | n.d. | Nonomura *et al.* [16]; |
| | Radiation | – | + | n.d. | Kangasniemi *et al.* [8]; Crawford *et al.* [17]; Wang and Meistrich, unpublished data |
| Rat | Radiation[a] | ++ | ++ | + | Meistrich and Kangasniemi [33]; Kurdoglu *et al.* [57] |
| | Procarbazine[a] | ++ | ++ | n.d. | Delic *et al.* [18]; Morris and Shalet [19]; Jégou *et al.* [20]; Parchuri *et al.* [21]; Meistrich *et al.* [37] |
| | Busulfan | – | + | n.d. | Udagawa *et al.* [38]; Udagawa *et al.* [58] |
| | DBCP | | ++ | + | Meistrich *et al.* [40] |
| | Aging | | | + | Schoenfeld *et al.* [59] |
| Monkey | Radiation | – | – | n.d. | Boekelheide *et al.* [13]; Kamischke *et al.* [31] |
| Human | Chemotherapeutic drug combinations (± radiation) | –/? | | – | Johnson *et al.* [48]; Waxman *et al.* [49]; Redman and Bajorunas [50]; Fossa *et al.* [51]; Kreuser *et al.* [52]; Thomson *et al.* [55] |
| | Radiation | – | | n.d. | Brennemann *et al.* [53] |
| | Cyclophosphamide | ++ | | n.d. | Masala *et al.* [54] |

DBCP, dibromochloropropane.
[a] Instances in which the type A spermatogonia showed a block in differentiation.
+, ++, relative effectiveness at protection or stimulation.
–, no protection/stimulation.
?, no protection observed but could not be demonstrated or ruled out with experimental design.
n.d., the effect not tested.

Experimental studies, particularly in rodents, are of great value in that they may be highly controlled, have larger sample sizes and can be used to optimize treatments and to elucidate mechanisms. Primate studies have greater variability and uncertainties and sample sizes are limited. The main question is what aspects of the rodent studies are applicable to the human and what aspects are not. Since primates and rodents diverged 66 million years ago (Mya) there will be differences. It is also noteworthy that mouse and rat diverged 41 Mya, whereas humans and macaques diverged 23 Mya. Thus, significant differences are expected among rodents and between rodents and different primates. It is important to understand the mechanism of protection or stimulation of recovery by hormonal suppression in order to determine which individual steps in the process will be similar or different between rodents and primates.

Cytotoxic agents which spermatogenesis is sensitive to in both rodents and primates are appropriate to utilize in experimental studies for extrapolation to men. Mouse and human spermatogenesis are both sensitive to certain alkylating agents

(procarbazine, chlorambucil, busulfan) and radiation as measured by stem cell death and prolonged azoospermia, respectively [60]. However, there are also differences as spermatogonial stem cells in mice are sensitive to, and killed, by doxorubicin (Adriamycin) but not cyclophosphamide, whereas cyclophosphamide, but not doxorubicin, strongly induces long-term azoospermia in humans. It has to be noted that although both primate and mouse spermatogenesis are sensitive to radiation, the human [61–63] and monkey [64] appear be more sensitive to radiation than mice [65–67]. Thus, one of the directions of future research should be on elucidating the reasons for greater sensitivity of primate stem spermatogonia to radiation and certain chemotherapeutic agents in order to develop methods to protect them.

One contribution to the difference in the stimulation of recovery by hormone suppression after cytotoxic treatment may be the interspecies differences in the block in differentiation of spermatogonia. In rats exposed to moderate doses of cytotoxic agents, the induction of a block in spermatogonial differentiation is a much more likely cause of prolonged azoospermia than is spermatogonial stem cell death. The reversal of this block in spermatogonial differentiation appears to be the mechanism by which hormone suppression protects or restores spermatogenesis in toxicant-exposed rats [27], and the occurrence of such a block should indicate whether hormonal suppression might stimulate spermatogenic recovery in another species. Although, in many cases, the seminiferous tubules in testicular biopsies taken from men with chemotherapy or radiotherapy-induced azoospermia contain only Sertoli cells and no spermatogonia [68], occasionally the presence of isolated spermatogonia have been observed at relatively long times after chemotherapy treatment [69]. In addition, spontaneous recovery of spermatogenesis in some men more than 1 year after radiation [70] or chemotherapy [71] also implies a block in the differentiation of spermatogonia that survive these cytotoxic exposures. These results suggest that after some cytotoxic therapy regimens, there is a potentially reversible block to spermatogonial differentiation in men. It should be noted that there is no evidence of a similar spermatogonial block in monkeys [13]. The human data emphasize that the cytotoxic therapy regimens need to be carefully selected in these clinical trials from a range of regimens. To have a chance of success, doses of cytotoxic therapies should be chosen at which there is an appreciable block to spermatogonial differentiation but not the killing of all stem spermatogonia.

Since many chemo and radiotherapeutic regimens may result in the complete killing of the stem spermatogonia and the hormonal methods do not protect these cells from cytotoxicity, consideration should be given to the applications of hormonal suppression in combination with spermatogonial transplantation. Cryopreservation of spermatogonia and autologous transplantation is considered a potential method for restoring spermatogenesis and possibly rescuing fertility after chemotherapy or radiotherapy [72]. Hormonal suppression could restore the somatic environment in human testes sufficiently to promote the ability of transplanted stem spermatogonia to develop, as was the case with rat testes [12].

It is also important to consider whether the molecular and cellular effects of hormonal suppression are similar in humans and in rats. Although the basic processes by which GnRH analogues suppress gonadotropin and testosterone levels and induce a block in the completion of spermatogenesis in normal adult males are similar in rodents and primates, there are quantitative differences. Whereas in rats and humans, GnRH antagonist reduced intratesticular testosterone concentrations to about 2% of that observed in controls [73, 74], in macaques it only reduced intratesticular testosterone concentrations to 28% of control [75]. Despite the less marked reduction in intratesticular testosterone levels, spermatogenesis was blocked at the B spermatogonial stage by GnRH antagonist treatment of the primates [75] compared to the round spermatid stage in rats [8]. In humans, the block in spermatogenesis was also largely at the B spermatogonial levels, but later germ cells to the round spermatid stage were still produced at 20% of control levels [74]. The restimulation of spermatogonial differentiation by hormonal suppression in rats may be dependent upon the ability of germ cells to develop to the spermatocyte stage during the testosterone suppression, and hence might occur in men. Further germ cell differentiation in the presence of suppressed testosterone can be induced in human testes by treatment with FSH [76].

Thus, the mechanism by which hormone suppression protects or reverses the damage to the somatic elements of the testis to stimulate spermatogenic recovery in rodents is not yet clear. Knowledge of the mechanism in appropriate animal models should allow the identification of treatment targets downstream from

the initial action of the hormones. It may be possible to suitably modulate those targets in primates to release the blockade in spermatogenic differentiation, while maintaining hormone levels, which should allow spermatogonial differentiation. In addition, elucidation of mechanisms for greater sensitivity of primate germ cells to cancer therapeutic agents and development of ideal conditions for germ cell transplantation in primates may open strategies to recover spermatogenesis and sperm count in toxicant-exposed human males.

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Chapter 14

# Cryopreservation of spermatozoa

## Old routine and new perspectives

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## Introduction

Cryopreservation of male and female gametes has been long established, and nowadays low-temperature storage of human spermatozoa is a routine technique in assisted reproduction. This technique offers the following advantages over the use of fresh ejaculated spermatozoa: (1) storage of both homologous or donor sperm for subsequent intrauterine insemination, in vitro fertilization (IVF) and intracytoplasmic sperm injection; (2) long-term storage of known quality donor semen; (3) the ability to quarantine donor semen until appropriate testing can be completed; (4) preservation of epididymal or testicular spermatozoa/tissue for subsequent intracytoplasmic sperm injection or necessary diagnosis [1, 2]. Recently, fertility preservation has been a "hot topic" and attracted much attention because chemotherapy and radiotherapy result in a significant reduction of spermatozoa quality and, as a consequence, a following indefinite period of infertility [3–5]. Human genome banking is one specific approach that can be used to preserve male genetic material before sterilization, chemotherapy or radiotherapy. It can also be used for males with autoimmune diseases [2, 4, 6–8] or before some treatments and special kinds of surgical procedures which may lead to testicular failure or ejaculatory dysfunction [9].

The empirical methods of cryopreservation developed in the 1950s are still used today [10, 11] and based on use of a relatively slow cooling rate (1–170°C/min) in a region of critical temperatures (–10° to –60°C). At present the major steps used for cryopreservation of different kind of cells can be summarized as follows: (1) Adding cryoprotective agents (CPAs) before freezing. This substance enhances post-thaw survival by limiting the crystallization of water. (2) The seeding of samples at freezing or around freezing point. (3) The thawing of the cells. (4) Removing the CPAs from the cells after thawing [12]. Cells can endure storage at very low temperatures (below –100°C). However, the cooling and warming processes associated with the intermediate zone of temperature (–10°C to –60°C), which cells must traverse twice – once during cooling and once during warming – can be lethal to cells [11]. The aim of slow cooling rates is to maintain a very delicate balance between ice crystal formation and the growing concentration of dissolved substances. The danger is cell damage due to crystallization of intracellular water and osmotic and chilling injury; cytoplasm fracture; or even effects on the cytoskeleton, genome or genome-related structures [13–16].

Despite these dangers, the use of programmable or non-programmable conventional slow freezing allows the simultaneous preservation of relatively big volumes of diluted ejaculate or prepared spermatozoa, from 0.25 to 1.0 ml [17–19], with satisfactory results based on the motility of spermatozoa after thawing [20–22], the integrity of the acrosomal and cytoplasmic membrane [23], the functional activity of mitochondria [24, 25], DNA stability [26, 27] and the prevention of phospholipids translocation inside the spermatozoa membranes [28, 29].

## Vitrification: a promising new direction

The success of these current procedures brings up the reasonable question: "Why do we need to develop other technique and what advantages will it give us compared to the current system?" This "new" technique is vitrification and it could be beneficial

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compared with the conventional slow freezing method. The vitrification method uses no specially developed cooling program; it does not need to apply permeable cryoprotectants; it is much faster, simpler and cheaper; and it can also provide a high recovery of motile spermatozoa after warming as effective protection of spermatozoa against cryodamage [30–33]. The method is based on the cooling of the cells by direct immersion into liquid nitrogen ($LN_2$), thereby avoiding the formation of big intracellular ice crystals [34]. In routine work, the vitrification method – using high concentrations of permeable cryoprotectants – was successfully applied in 1985 for mouse embryos [35], and at present is successfully used for the preservation of female gametes [36, 37] and embryos [38, 39]. However, to apply this protocol to spermatozoa cryopreservation is impossible because of the resulting osmotic and cytotoxic effects [13, 40]. The promising results after successful vitrification of frog [41] and fowl [42] spermatozoa were not confirmed in subsequent investigations [43, 44], and work in this direction stopped for 40 years. At present, as an extremely rapid method of cryopreservation [45], vitrification is being investigated extensively and applied to embryos [46] and oocytes [26, 47] but very seldomly to spermatozoa, with the exception of a few reports [26, 27, 30, 32, 33, 48–53].

At present, preservation techniques for natural diversity fall into two categories: (1) *in-situ preservation* – the preservation and protecting of individual species in their natural setting; and (2) *ex-situ preservation* – the preservation of individual species genomic material in combination with breeding programs [54]. In human medicine, the cryopreservation of male gametes offers both men and boys the possibility of preserving their fertility. The general creation of cryobanks, for the low-temperature storage of genetic materials, came after the discovery of the beneficial effects of glycerol and non-permeable cryoprotectants on plant cryostability [55]. Subsequent investigation of glycerol's properties applied to mammalian spermatozoa supports these results [56–59]. These empirical methods, which were subsequently developed in the 1950s for use in many species and still applied today, have opened a new era of permeable cryoprotectants. However, before now, the technology for sperm cryopreservation did not provide complete protection of the motility of cryopreserved/thawed cells; motility dropped to about 50% of their prefreezing value, with considerable intersample fluctuation taking place [13, 60]. The question of diminished spermatozoa motility after cryopreservation is crucial since this variable is known to be the first affected by cryopreservation [61], although the mechanism of sperm impairment and its mechanical and/or physical–chemical etiology remains unclear. The reason for cell damage could be due to mechanical cell injury, as a consequence of intracellular or extracellular ice crystal formation, and osmotic damage due to extensive cell shrinkage during conventional freezing. The warming process also has a negative influence on cells, possibly through excessive osmotic swelling [10, 62]. Both these factors accompany slow freezing due to the chemical and physical damage of the sperm cell membranes, which is the result of changes in lipid phase transition and/or increased lipid peroxidation and active production of reactive oxygen species [63] and the subsequent loss of sperm motility [24, 64]. The permeable and non-permeable cryoprotectants, used during conventional freezing to prevent intracellular ice formation, can be damaging due to the so-called CPAs' toxicity [30]. This toxicity includes damage to the cell's membrane due to the osmotic and chemical influence of CPAs on cells during freezing and/or thawing [62, 65], which activates an apoptosis-like mechanism [66] that can also lead to chromatin damage [67]. All of these findings suggest that, quite apart from ice crystal formation, slow cooling, especially the thawing of spermatozoa, is intrinsically deleterious.

Compared to conventional slow freezing, the vitrification technique, even though it has its own peculiarities, is a real alternative.

## How does vitrification work in principle?

Luyet wrote that crystallization is incompatible with living systems and should be avoided wherever possible [34]. However, he considered the cooling of small living systems at ultra-high speeds of freezing possible, and that this could eliminate big crystal formations and create instead a glass-like (vitreous) state [34]. This constituted the origin of the idea of vitrification but not, however, the beginning of the vitrification of organs, which was unthinkable at the speed of freezing and thawing demanded by Luyet [34]. It is known that vitrification is used as a natural form of cryoprotection in some arctic plants [68]. In contrast to slow-rate freezing protocols, during vitrification the entire solution remains unchanged and the

water does not precipitate, so no ice crystals are formed [69]. The physical definition of vitrification is the solidification of a solution (water is rapidly cooled and formed into a glassy, vitrified state from the liquid phase) at low temperature, not by ice crystallization but by extreme elevation in viscosity during cooling [70]. Fahy expressed this as follows: "… the viscosity of the sample becomes greater and greater until the molecules become immobilized and the sample is no longer a liquid, but rather has the properties of a solid" [69]. However, vitrification is a result of high cooling rates associated with high concentrations of cryoprotectant. Inevitably, this is biologically problematic and technically difficult [71].

There are two ways to achieve the vitrification of water inside cells:

1 To increase the speed of temperature conduction.
2 To increase the concentration of cryoprotectant.

In addition, by using a small volume of high concentration cryoprotectant (<1 μl), very rapid cooling rates of 15 000–30 000°C/min can be achieved (e.g. ΔT from –196°C to 25°C = 221°C/0.5 s = 5). The strategy of vitrification is realized by decreasing the volume of the ice crystals of extracellular water and the total elimination of the crystallization of intracellular water.

A practically glass-like solidification of solution is achieved by vitrification of the bulk solution without any sizable portions of ice during cooling and thawing (see our previous description [30]). This process is performed substantially faster than with equilibrium freezing (during which ice is formed). It is achieved by the combination of relatively slow to moderate speeds of cooling (up to 105°C/min) with the use of high concentrations (3.5–8.0 mol/l) of permeable CPAs, or by solidification of the bulk solution by abrupt cooling at a very high speed to temperatures below the glass-transition temperature of the solution by directly plunging the specimen into $LN_2$. Thus, the formation of a glassy state (with some molecule regulation) is accompanied by an extreme elevation in viscosity (proportional to the cooling rate) of pure water/solution during cooling. In the glassy state all physical–chemical processes are completely arrested. It is known that it is possible to provide cryoprotection of cells by alcohols (including glycols), amines (including amides), sugars, inorganic salts and macromolecules (including proteins and polysaccharides) and dimethylsulfoxide (DMSO) (polar aprotic solvent is a solvent that shares ion-dissolving power with protic solvents but lacks an acidic hydrogen and has a high dielectric constant and high polarity) [12]. However, it is also well-known that the fastest of all permeable CPAs (the most used such as ethylene and propylene glycol, glycerol, DMSO) have a toxic influence on living cells [72–74]. The toxic effect of highly concentrated permeable CPAs (critical CPA concentration, *Cv*) are possibly lowered by including some non-permeable CPAs, such as carbohydrates (saccharides), polymers (polyvinylpyrrolidone), polyols (polyethylene glycol), polysaccharide (Ficol), amines (acetamide, formamide), inorganic salts (sodium citrate, ammonium sulfate), proteins (albumin, antifreeze peptide/glycopeptide), phospholipids (hen egg yolk) or by using the combination of two or more permeable and non-permeable cryoprotectants [72]. As a rule, carbohydrates are used for sperm cryopreservation to compensate for the decrease in osmotic pressure caused by the permeable cryoprotectant glycerol, which works as an additional dissolvent and has the property to decrease the medium's osmotic pressure [75]. For example, the combination of sugars with permeable CPAs [76] has a major influence on the vitrification properties of such a cryoprotective mixture, resulting in a lowering of permeable CPAs toxicity to embryos and oocytes and a significant decrease in the concentration of permeable cryoprotectants needed for efficient cryopreservation [77]. In general, the incorporation of non-permeating compounds into the vitrifying solution, and the incubation of the cells in this solution before any vitrifying, helps to withdraw more water from the cells and to lessen the exposure time of the cells to the toxic effects of the cryoprotectants. This is not surprising as the sugars, as non-permeable cryoprotectants, possess unique properties: during the cell's dehydration they compensate for the osmotic pressure drop (acting as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage), simultaneously stabilizing the cell's membrane [48, 78–80]. Cells naturally contain high concentrations of protein, which is helpful in vitrification. Higher concentrations of cryoprotectants are needed for extracellular than for intracellular vitrification. It was demonstrated that in certain circumstances a polymer can reduce the *Cv* on average by 7%, and by as much as 24% in combination with increased hydrostatic pressure [70]. Early studies evaluated the potential beneficial effects

of adding macromolecular solutes to the vitrification solution to facilitate vitrification [81]. These polymers can protect embryos against cryo-injury by mitigating the mechanical stresses that occur during cryopreservation [82]. They do this by modifying the vitrification properties of these solutions by significantly reducing the amount of cryoprotectant required to achieve vitrification itself [83]. They also influence the viscosity of the vitrification solution and reduce the toxicity of the cryoprotectant through lowered concentrations.

Furthermore, the polymers may be able to build a viscous matrix for the encapsulation of cells, and also prevent crystallization during cooling and warming [81]. Indeed, O'Neill observed that addition of polyethylene glycol (PEG) resulted in greatly improved viability of oocytes following cryopreservation, and vastly reduced the variability seen with vitrification solution alone [84]. The possible toxic effect of permeable CPAs was reduced by exposing the cells to a graded series of pre-cooled concentrated solutions. The combination of these described methods and increasing the rate of cooling and warming reduces both the toxic and osmotic effects of cryoprotectants.

The higher cooling rate may be provided by use of an electron–microscopical grid [85]; or by a nylon loop allowing an effective reduction in the concentration of single permeable cryoprotectant; or by substituting the single permeable cryoprotectant with a mixture of permeable cryoprotectants [40]. The small amount of such cryopreservation solution (0.1–1.0 μl) immersed directly into $LN_2$ assists in achieving the maximal cooling rate. The optimal cooling rate is achieved with the following specially designed packaging systems: Open Pulled Straws [86]; the Flexipet denuding pipette [40]; micro-drops [87]; electron microscope copper grids [88]; the Hemi-straw system [89]; the Cryotop [90]; small nylon coils [91] or nylon meshes [92]; the Cryoloop [93]; the Cryoleaf [94]; the Cryotip [95] and some others. All of these packaging systems have been successfully used in routine human and veterinary reproductive medicine for the vitrification of oocytes and embryos [47, 96–101]. In our experience, similar results can be achieved in the absence of the "conventional" CPAs provided that the cooling/warming speed is high enough to ensure both intracellular and extracellular vitrification [30, 33]. In general, the rate of cooling/warming and the concentration of CPAs required to achieve vitrification are inversely related. In other words, the faster the cooling and warming is undertaken, the lower the critical solute concentration necessary to obtain ice-free vitrification [34, 41, 77]. Given the biological and physiochemical effects of cryoprotectants and the high concentrations used in vitrification, it is not surprising that cryoprotectant toxicity has been described as a key limiting factor in the cryobiology of vitrification [34, 41]. In an attempt to avoid this toxicity, we achieve ultra-rapid cooling and warming rates, in the range of 0.5–1.0 $\times$ $10^6$K/min, using a relatively dilute vitrification medium (around 12% total solute content) of similar solute concentration to semen or blood; thus, "resurrecting" the original vitrification approach of Luyet.

## Spermatozoa vitrification techniques

The era of vitrification began with the famous work of Luyet [34]. The success of Luyet's vitrification technique was supported by Shaffner applying the technique to frog spermatozoa after vitrification of fowl sperm [42]. However, the subsequent applying of this technique to the spermatozoa of different kind of animals, including humans, showed non-promising results: spermatozoa survival was very low or lacking [43, 44]. The problem was the low concentration of CPAs tolerated by sperm at high speed. Active investigation in this direction stopped for 25 years, and it only began again in 1980 following the fundamental work of Rall and Fahy [35]. They discussed vitrification of mouse embryos by application of high CPA concentrations and a relatively low speed of cooling and warming. It opened new horizons for applying this technique to different kind of cells, including tissues from different living organisms. The main approach to vitrification of spermatozoa is the same as used for other types of mammalian cells [102]. However, it was impossible to completely extrapolate this technique to male gametes, due to the lethal osmotic effects and possible chemical alterations following use of highly concentrated permeable cryoprotectants in combination with a high mode of cooling (2000–30 000°C/min).

Indeed, applying "conventional" methods of vitrifying to human spermatozoa resulted in very low or null spermatozoa survival rates. This prompted the idea of exploring vitrification methods that would not require high concentrations of potentially toxic CPAs. Seventy years of cryobiology experience had showed

**Table 14.1.** The relationship between the size (approximate) of mammalian sperm heads and stability of gametes to cold shock

| | Boar | Bull | Ram | Rabbit | Cat | Dog | Stallion | Human |
|---|---|---|---|---|---|---|---|---|
| Length (μm) | 9.0 | 9.1 | 8.1 | 7.7 | 7.7 | 7.0 | 6.5 | 4.6 |
| Maximal width (μm) | 5.0 | 4.7 | 4.0 | 4.5 | 3.2 | 5.0– | 3.4 | 3.2 |
| Area of projection ($\mu m^2$) | 37.5 | 34.2 | – | 28.0 | – | + | 15.2 | 10.8 |
| Sensitivity of spermatozoa to cold shock | ++++ | +++ | +++ | ++ | + | | + | + |

From Watson and Plummer [107] with permission.

that cryoprotectants are not always needed for successful vitrification. The first investigations in this direction had very contradictory results. Luyet, the pioneer in vitrification investigation, showed that a small specimen cooled very rapidly could be vitrified without substantial loss of viability [34]. There followed subsequent investigations by Jahnel [103] and then Parkes [104] in which they performed cryoprotectant-free cryopreservation of human spermatozoa cooled in $LN_2$ and liquid helium (−269.5°C) using glass or metal tubes as packaging with a large volume of sperm suspension (a few milliliters). They did not get such promising results, seemingly due to the difficulty of achieving a quick enough mode of warming. All subsequent attempts to vitrify mammalian spermatozoa using this approach resulted in low or null survival [43, 44], mostly because of the critical speed of freezing and warming, which is very high for low concentrations of cryoprotectants. Such situations were why it was considered that the vitrification could only be achieved using high concentrations of permeable cryoprotectants alone or in combination with non-permeable cryoprotectants [105]. However, classical vitrification requires a high percentage of permeable cryoprotectants in medium (30–50% compared to 5–7% with slow freezing) and is unsuitable for the vitrification of spermatozoa due to the lethal osmotic effect [65, 70]. It was reported that, by optimizing regimens for the addition and removal of the CPAs, it may be possible to significantly reduce the damaged effect of these substances on male gametes during the equilibration time before freezing [13, 62, 65, 106]. But, based on numerous publications dedicated to this theme, it must be emphasized that generally, in the majority of species, sperm cannot tolerate the high concentrations of cryoprotectants conventionally used for vitrification. One peculiarity of male gametes could be a significant factor: the shape and size of the sperm head, which defines the cryosensitivity of the cell. Comparative studies on various mammalian species (boar, bull, ram, rabbit, cat, dog, horse, human) showed a negative correlation between the size of the sperm head and cryostability (Table 14.1 [107]) [108]. Among the above-mentioned species, human spermatozoa possessed the smallest size with maximal cryostability [10]. Taking all of this into account, we believed that vitrification of human spermatozoa with no or a low amount of permeable cryoprotectants, in combination with a relatively small sample size (from a few to hundreds of microliters), could be successful. Such a combination would achieve a very quick mode of cooling and warming. This was theoretically predicted in 2000 by Bischof's group [106]. They wrote that one of the optimal cooling rates for spermatozoa theoretically lies between 5000 and 7000°C/min. A few years later, we were able to prove this theoretical prediction. In our first studies, we demonstrated the possibility for successful vitrification of human spermatozoa without permeable cryoprotectants, using 1% of human serum albumin (HSA) as the chosen non-permeable cryoprotectant [30, 33]. The chosen package system was self-manufactured cryoloops. To investigate the effectiveness of this method of cryopreservation, we compared it with conventional slow freezing. Comparison of the physiological parameters of spermatozoa following cryopreservation was done using split-sperm samples. For cryopreservation of spermatozoa, suspension was performed using the vitrification method without permeable cryoprotectants using cryoloops as the package system. Following this, a sample of spermatozoa suspension (20 μl volume of drops) was placed onto a copper loop (5 mm diameter) and then directly plunged into $LN_2$. The warming of the cryoloop was performed by plunging the cryoloops into a centrifuge tube with 10 ml of sperm-preparation medium at 37°C under intense agitation. The routine conventional slow freezing was done using French straws [109] and standard cryoprotective solution (TYBG,

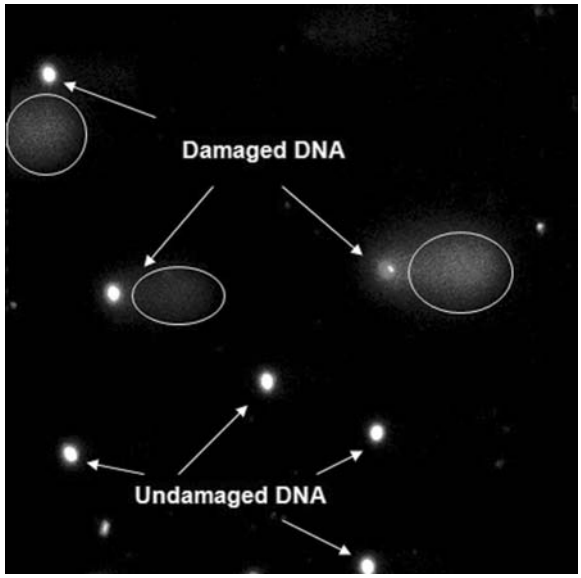


**Figure 14.1** Damaged and undamaged DNA after warming. Fluorescent staining was performed using SYBR green stain (working concentration 1:200). In healthy cells, the fluorescence was confined to the nucleoid: undamaged DNA is supercoiled and does not migrate very far from the nucleoid. In cells that have incurred damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the nucleoid and form the so-called "comet-tail" (circled). See plate section for color version.

Scandinavian IVF Science, Sweden). The swim-up prepared spermatozoa, conventionally frozen with cryoprotectants immediately after thawing, showed 38% motility compared to 49% after vitrification without cryoprotectants. The number of morphologically normal spermatozoa after applying both cryopreservation methods was not significantly different (27% for slow frozen with permeable cryoprotectants versus 26% for vitrified without permeable cryoprotectants, $P > 0.01$). Programmable freezing in the absence of cryoprotectants dramatically reduced all sperm variables.

The negative influence of freezing on cells can lead to chromatin damage and is strongly correlated with mutagenic effects [23]. It has been shown that there is a correlation between fertilization rates in IVF [110] and intracytoplasmic sperm injection (ICSI) [111] with fragmented DNA. We performed the comet assay to investigate the occurrence of apoptosis in spermatozoa DNA after vitrification (Figure 14.1). Our albumin estimates [26] showed that devitrification (especially intracellular) did not occur [112] during cooling or, especially, during re-warming/resuscitation, due to the small amount of specimen and cells, the high viscosity of the solution and the high speed of cooling and warming [30]. Our investigation showed that no significant difference in DNA integrity was found, independent from the mode of cooling and the presence of cryoprotectants ($P > 0.05$) (Figure 14.2) [26]. We found that the DNA integrity of vitrified spermatozoa was comparable with standard slow frozen and thawed spermatozoa. The DNA integrity ($P > 0.05$) was found to be unaff1ected by the vitrification method. Our results supported the results of the Schrader group [113] and Duty [114]. They found no difference in sperm chromatin structure assay results for cryopreserved or non-cryopreserved sperm, or for slowly or flash-frozen specimens [113]. They suggested that the unique packaging of sperm DNA protects it from intracellular fluid shifts and the formation of the nuclei of crystallization during the cooling–warming cycle. The use of spermatozoa vitrified using this method for IVF of human oocytes resulted in successful fertilization and development to the blastocyst stage [33].

The next modification of the vitrification method was the direct dropping of spermatozoa suspension into $LN_2$ (Figure 14.3 [115]) [32, 49]. Immediately following vitrification, 30 μl of sample was dropped directly into $LN_2$. The temperature of the foil was determined by an electrical thermometer. After 5 min of cooling, the solution-precursor-solid (SPS) droplets were placed into pre-cooled $LN_2$ Cryovials and stored there until the time of use. The warming of the solid sphere was performed by directly plunging it into a centrifuge tube with 10 ml of sperm-preparation medium (SPM) at 37°C under intense agitation.

Based on our previous data and the peculiarities of carbohydrates, we decided to investigate the contribution of disaccharide sucrose to sperm cryoprotection during cooling/warming using the dropping method. The investigation focused especially on the ability of sucrose to support the motility of spermatozoa, prevent the artificial induction of capacitation or acrosome reaction and support mitochondrial stability during vitrification/warming. The viable, acrosome reacted and capacitated spermatozoa (Figure 14.4) were detected using the double-fluorescence Chlortetracycline (CTC)-Hoechst 33258 staining technique [116].

The results have shown that the medium supplemented with 1% HSA + 0.25 M sucrose allowed the motility rate to increase after warming to 57%, compared to HSA alone (45%) or pure medium without

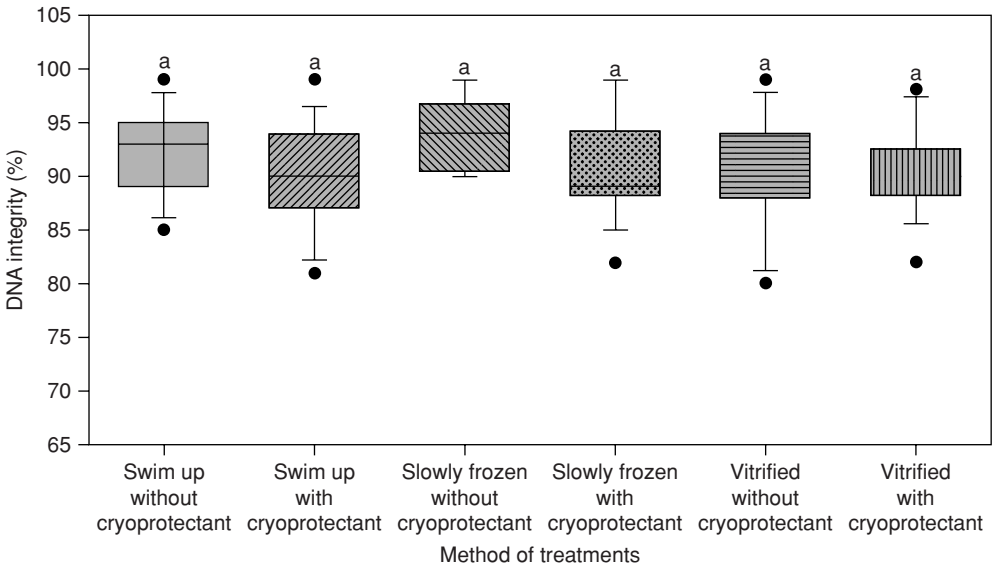


**Figure 14.2** DNA integrity of spermatozoa according to different treatments and cryopreservation methods. Each bar represents the medium, 25 and 75 percentile, minimum and maximum values. Bars with different letters inside of each treatment group represent significant differences ($P < 0.05$). With permission from Isachenko *et al.* [26].

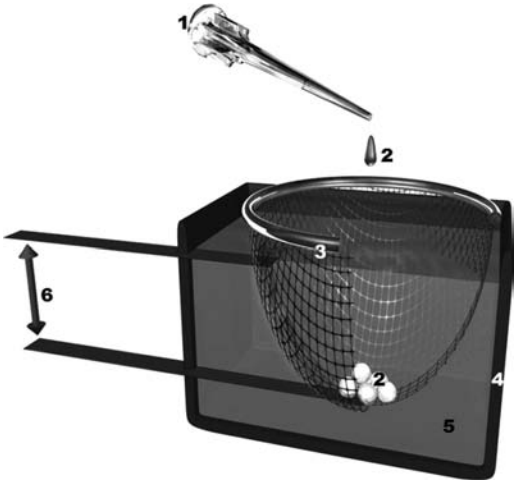


**Figure 14.3** Scheme of the spermatozoa vitrification procedure. (1) Single channel pipettes with adjustable volume (30 μl). (2) Spermatozoa suspension. (3) Strainer. (4) Foam box. (5) Liquid nitrogen. (6) Distance between bottom of strainer and surface of liquid nitrogen (minimum 3 cm). With permission from Isachenko *et al.* [115]. See plate section for color version.

cryoprotective substances (19%) (Figure 14.5a) [32]. The medium supplemented only with HSA provided 21% motility of spermatozoa after 24 h of culture compared to 2.5% motility with non-supplemented medium. The combination of two HSA and sucrose non-permeable substances in the cryopreservation medium provides 32% motility of spermatozoa after 24 h of culture. Our data showed that the percentage increase of capacitated spermatozoa after warming was not significantly different to controls in both groups (HSA: 7%; HSA + sucrose: 11%; control: 9%; $P > 0.1$). The acrosome-reacted spermatozoa in the group with both supplements (HSA and sucrose) were detected in 6%, in the group with one supplement (HSA) in 11% and in the control in 10% ($P > 0.05$). The physiological condition of spermatozoa, such as capacitation and acrosome reaction, was not affected by the composition of cryoprotective medium (Figure 14.6) or by the vitrification technique (Figure 14.5b). The results of our investigation corresponded to data of Esteves *et al.* [117]. It showed no statistical difference between numbers of capacitated and acrosome-reacted spermatozoa of control and experimental groups. According to the data, the acrosome reaction after cryopreservation may involve some complex mechanisms rather than a physiological change induced by capacitation. However, at present, routine conventional freezing for human sperm cryopreservation does not induce the capacitation process or acrosome reaction but also does not sufficiently preserve mitochondrial integrity [24, 25].

To evaluate mitochondrial activity, we measured changes in the mitochondrial membrane

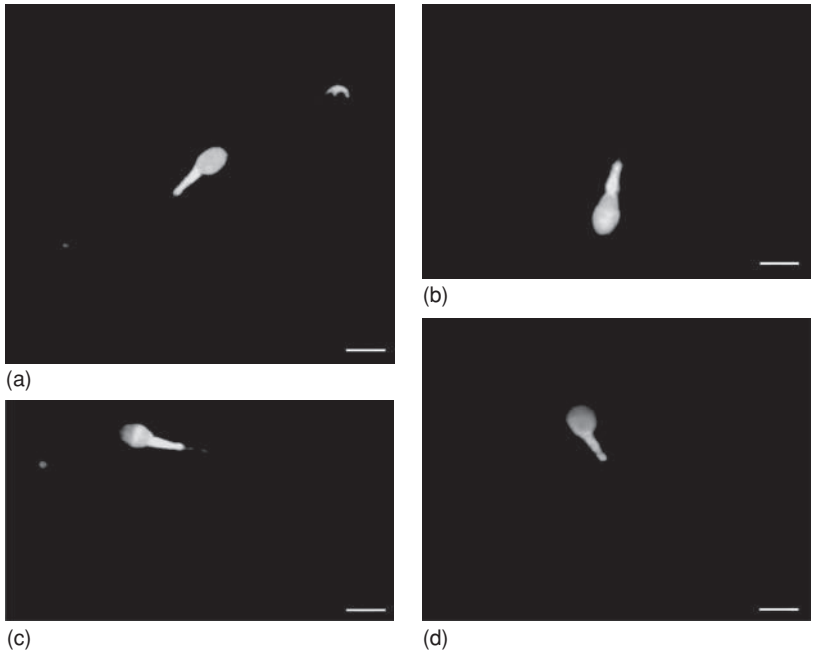


**Figure 14.4** Example of non-capacitated (a), capacitated (b), acrosome-reacted (c) and non-viable (d) spermatozoa. At least 200 spermatozoa were observed in each plate and 3 patterns were identified (see Figure 14.2). (a) A uniform fluorescence on the head of the spermatozoa (non-capacitated spermatozoa). (b) A band of fluorescence diminished in the post-acrosomal region and a relatively shining fluorescence in the acrosomal region (capacitated spermatozoa). (c) A fluorescence in the complete head of the spermatozoa, except a tenuous band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa). The slides were viewed using a Zeiss Axiolab Epifluorescence microscope that was equipped with an excitation/emission filter of 485 nm/520 nm under × 400 magnification. The non-viable spermatozoa were observed with the filter set 09 (450–490 nm). The dead spermatozoa displayed a pattern of blue fluorescence in the whole head (Figure 14.2d). With permission from Isachenko *et al.* [115]. See plate section for color version.

potential (M $\Delta\Psi$) using a unique fluorescent cationic dye, 5,5′, 6,6′-tetachloro-1–1′, 3,3′-tetraethylbenzamidazolocarbocyanin iodide (Figure 14.7), commonly known as JC-1 [118]. This test was performed as per manufacturer's instructions for Mitochondrial Permeability Detection Kit AK-116 (MIT-E-$\Psi^{TM}$, BIOMOL® International LP, Plymouth Meeting, PA) and applied for the early detection of the initiation of cellular apoptosis. However, the mature healthy human ejaculated spermatozoa are incapable of undergoing apoptosis [119]; they lack the necessary assemblage of enzymes and signaling pathways possessed by cells capable of undergoing this process, and the lack of orange-red fluorescence due to binding of this cationic probe by the sperm mitochondria implies only the loss of energy conservation capacity. The mitochondria are most probably uncoupled rather than membrane damaged; but uncoupled mitochondria are evidence of unhealthy sperm and so this determination is a useful one to document post-thaw sperm survival by determination of mitochondrial activity. It has previously been demonstrated that mitochondrial activity and viability are equally susceptible to cryopreservation-induced damage [25]. It has been suggested, however, that current conventional freezing techniques for human sperm cryopreservation are good enough to preserve all these structures [24, 25]. The non-permeable cryoprotective substances also showed stable cryoprotective effect on the mitochondrial membrane (Figure 14.5c). It was observed that the medium alone has no cryoprotective effect (8%). The combination of HSA and sucrose is more effective then addition of HSA only (65 and 33%, respectively; $P < 0.05$).

Unfortunately, these methods have a large technological disadvantage: their techniques do not prevent direct contact with $LN_2$. At present, numerous publications exist about microbial contamination of the different kinds of biological material cryopreserved in $LN_2$ [120–123]. Because of this, a special kind of

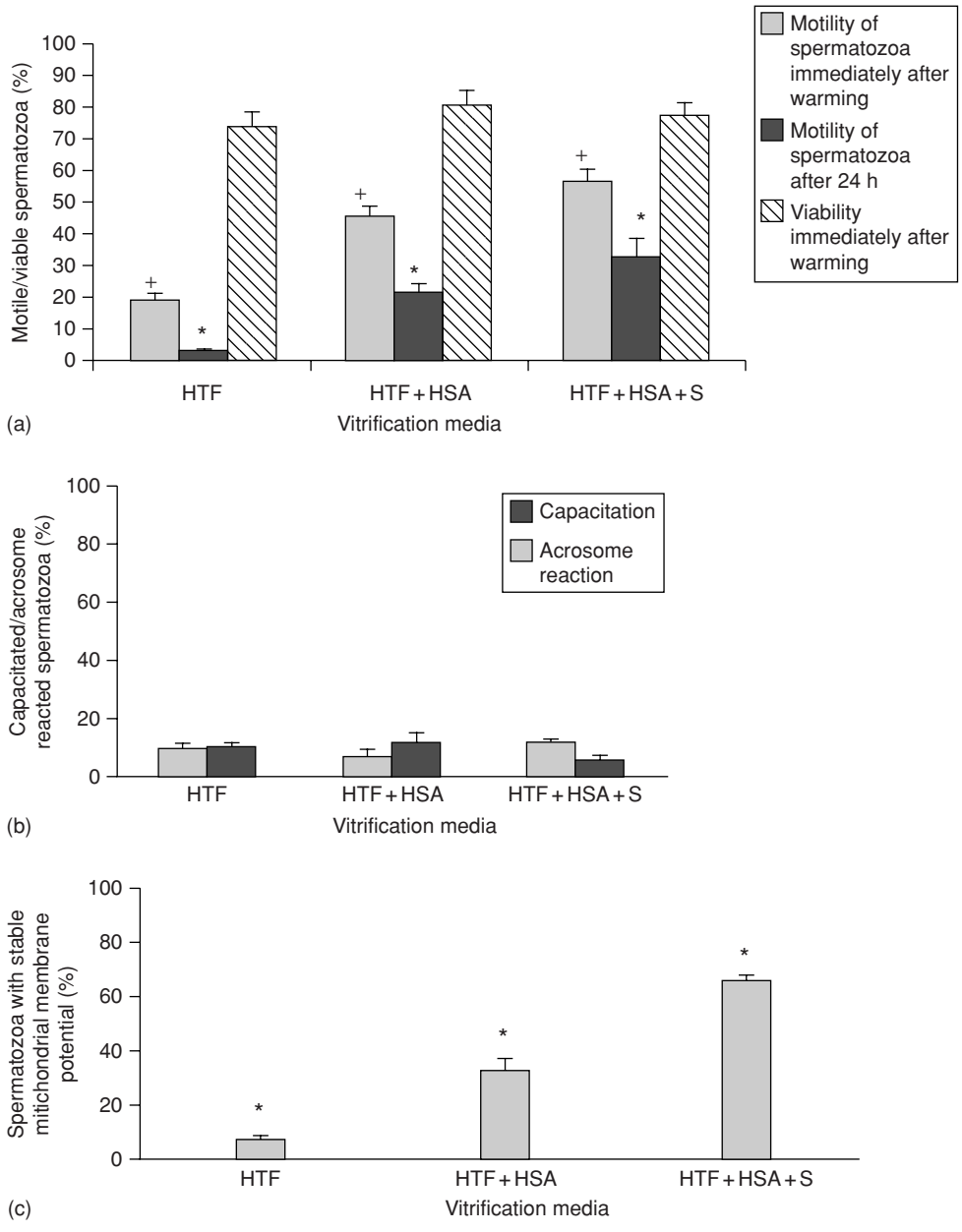


**Figure 14.5** (a) Cryoprotective effect of human tubal fluid (HTF) alone or in combination with human serum albumin (HSA) or with HSA + sucrose on motility and viability of vitrified spermatozoa. (b) Cryoprotective effect of HTF alone or in combination with HSA or HSA + sucrose on activation of capacitation and induction of acrosome reaction of vitrified spermatozoa. (c) Cryoprotective effect of HTF alone or in combination with HSA or HSA + sucrose on the mitochondrial membrane integrity of vitrified spermatozoa. Asterisks denote statistical differences between respective values of compared groups ($P < 0.05$). With permission from Isachenko *et al.* [115].

package system needed to be developed, one that allowed the biological material to separate from $LN_2$. However, caution was needed as the package system can significantly reduce the speed of cooling, resulting in undesirable results after warming. Therefore, it was necessary to try and develop an aseptic vitrification technique that prevented direct contact with $LN_2$. To do this, it was necessary to discover if the cooling or warming rate of vitrification most affected the spermatozoa parameters. According to the famous

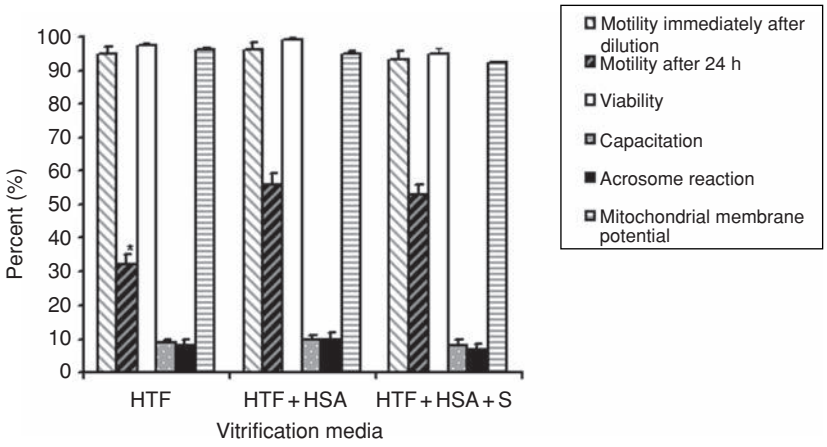


**Figure 14.6** Influence of human tubal fluid (HTF) medium alone or in combination with human serum albumin (HSA) or HSA + sucrose on motility, viability, activation of capacitation, induction of acrosome reaction and mitochondrial membrane integrity before cryopreservation. No statistical differences were found between the respective values of compared groups ($P > 0.1$) except for motility 24 h after in vitro culture ($P < 0.05$). With permission from Isachenko *et al.* [115].

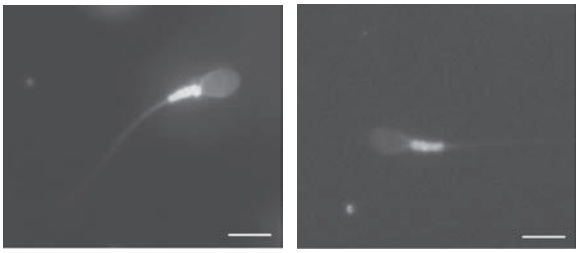


**Figure 14.7** Example of undamaged (a) and damaged mitochondria (b). In undamaged mitochondria the mitochondrial membrane potential (M $\Delta\Psi$) is intact and the JC-1 reagent aggregate inside of the non-damaged mitochondria and fluoresces red. In our case, the midpiece is yellow, as expected for the red fluorescence from JC-1 aggregates merging with the green fluorescence of JC-1 monomer dispersed throughout the cell plasma membrane. In damaged mitochondria, the M $\Delta\Psi$ is broken down and the JC-1 reagent disperses though the entire cells and fluoresces green. The changes in M $\Delta\Psi$ is measured using an unique fluorescent cationic dye, 5,5′, 6,6′-tetachloro-1–1′, 3,3′-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1. See plate section for color version.

investigations of Luyet, devitrification and the growth of ice crystals formed during cooling could be a key factor promoting cell damage during re-warming and thawing procedures [34, 41]. To accelerate the velocity of warming, we directly placed the specimens in a warm solution under gentle agitation, ensuring a very high rate of warming. This procedure prevents the substantial devitrification (re-crystallization) of the vitrified intracellular solution and an uncontrolled increase in the size of intracellular crystals due to the high speed and very short time of warming. Our estimations [30, 124] showed that during cooling and, especially, warming devitrification (especially intracellular) would not occur due to the following: (1) the high viscosity of the freezing medium and the small specimen size; (2) the high viscosity of the intracellular matrix and the small size of the cells, their low water content and their high degree of compartmentalization; and (3) the very high speed of warming (higher than the speed of cooling) [112, 125].

Our results [27] supported the data. The results showed that during vitrification applying a cooling rate of around 160–250°C/min in combination with very fast mode of warming (38°C) was enough to preserve ~60% motility. The results for the recovery of motile spermatozoa after "slow" vitrification was unstatistically different to spermatozoa cryopreserved using the "quick" vitrification method (Figure 14.8). DNA integrity was found to be non-affected by vitrification mode and was around 90%. The results from IVF show approximately equal fertilization potential for fresh human spermatozoa samples compared with swim-up prepared CPA-free samples vitrified by directly plunging into $LN_2$ or into nitrogen ($N_2$) vapor (Table 14.2). This is not surprising, because human spermatozoa contain large amounts of proteins, sugars and other components that make the intracellular matrix highly viscous and compartmentalized. The additional factor

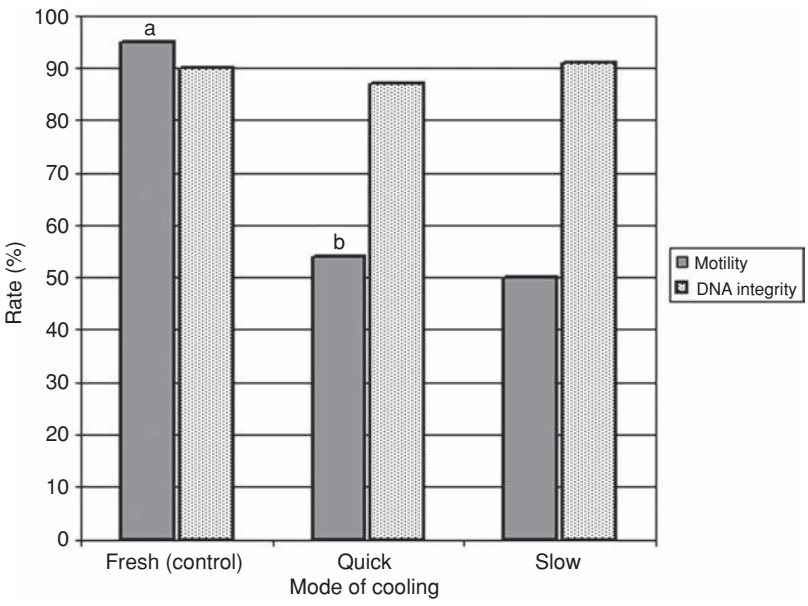


**Figure 14.8** The motility and DNA integrity of spermatozoa after cryoprotectant-free cryopreservation with quick (vitrification) and slow (freezing) cooling. There is no difference between the respective rates of vitrified and frozen spermatozoa ($P_{a-b} < 0.05$). With permission from Isachenko *et al.* [27].

that could be an influence on successful vitrification at such a low mode of cooling is the small size and high degree of compartmentalization of the sperm head. In such conditions, even if small (non-lethal) crystals start to form during this relatively "slow" cooling, there would be insufficient time for substantial growth during cooling. It is known that a major problem for such metastable systems is the regrowth of crystals and devitrification during warming. All of these findings led us to speculate the possibility that we could achieve intracellular vitrification of the human spermatozoa even at such a low rate of cooling. Guided by these results, we have two modifications of the aseptic technique (Figure 14.9) [49]. The first modification, aseptic cooling [49] in $LN_2$ using an Open Pulled Straw (Figure 14.9a) was performed as follows: 5–10 μl of spermatozoa suspension was drawn inside Open Pulled Straws by capillary effect [86]. The straw was located inside of a sterile 0.5 ml insemination straw, hermetically sealed with an ultrasound hand-held sealer and plunged into $LN_2$.

The second modification (Figure 14.9b), aseptic cooling in $LN_2$ using an insemination open straw [49], was performed as follows: 1–2 μl of sample was located on the inner wall, not far from the end of an insemination straw. For the same purpose, it is also possible to use the Cut Standard Straw (CSS) (Figure 14.10), which is made from a standard insemination 0.25 ml straw cut at an angle ~45° [77]. The straw is placed inside a sterile 0.5 ml insemination straw and sealed the same way as for the Open Pulled Straw method. The open pulled or insemination straws are rapidly warmed by immersion in 1.5 ml microcentrifuge tubes containing 1 ml of SPM at 37°C after being expelled from their packaging.

Comparative investigation of these four modifications of the vitrification technique showed that all cryopreservation regimens give about a 40% reduction in spermatozoa motility in comparison with non-treated swim-up control. No statistically significant difference was found in these parameters between all the regimens of cryopreservation tested [49].

Recently, the CSS aseptic vitrification technique was applied to ejaculate containing $13 \times 10^6$ motile spermatozoa/ml, <50% progressive motile and <30% morphologically normal spermatozoa [126]. The described methodology was finalized in Chile and approved by the University of Temuco de La Frontera, Ethics Committee. For vitrification, human tubal fluid (HTF) with 1% HSA and 0.25 M sucrose in the end-concentration was applied [127]. The 0.5 M sucrose was prepared in bi-distillate water with 1% HSA filtrated and frozen until use. After dilution, the cell suspension was maintained at 37°C in 5% $CO_2$ for 5 min before the cooling procedure was performed. Then 10 μl of spermatozoa suspension was deposited on the end of the inner part of a CSS, packaged into sterile 0.5 ml insemination straws, hermetically

**Table 14.2.** Fertilization properties of spermatozoa after cryoprotectant-free cryopreservation with "rapid" and "slow" cooling: results of in vitro fertilization and culture

| | After fresh spermatozoa (control) | | | | After vitrification with quick cooling | | | | After freezing with slow cooling | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Total oocytes (*n*)** | **Oocytes (*n*)** | **16 h, 2 PN and 3 PN (*n*)** | **48 h, 4–6 blastomeres (*n*)** | **56 h, EB and BL (*n*)** | **Oocytes (*n*)** | **16 h, 2PN and 3PN (*n*)** | **48 h, 4–6 blastomeres (n)** | **56 h, EB and BL (*n*)** | **Oocytes (*n*)** | **16 h, 2PN and 3PN (*n*)** | **48 h, 4–6 blastomeres (*n*)** | **56 h, EB and BL (*n*)** |
| 4 | 2 | 2 | 2 transfer | – | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | 3 | 2 | 2 transfer | – | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 0 |
| 8 | 4 | 3 | 3 transfer | 0 | 2 | 2 | 1 | 0 | 2 | 2 | 2 | 2 |
| 10 | 5 | 4 | 3 transfer | 1 | 2 | 1 | 1 | 1 | 3 | 2 | 1 | 1 |
| 12 | 5 | 5 | 3 transfer | 1 | 3 | 2 | 2 | 2 | 4 | 4 | 3 | 1 |
| 12 | 6 | 4 | 3 transfer | 0 | 3 | 3 | 1 | 1 | 3 | 3 | 2 | 1 |
| 13 | 6 | 4 | 3 transfer | 0 | 3 | 2 | 2 | 1 | 4 | 3 | 2 | 1 |
| 15 | 8 | 7 | 3 transfer | 2 | 3 | 2 | 2 | 2 | 4 | 3 | 2 | 2 |

(2PN) oocytes with two pronuclei; (3PN) oocytes with three pronuclei; (EB) early blastocysts; (BL) blastocysts.
No differences between spermatozoa vitrified using quick and slow cooling ($P > 0.01$).
With permission from Isachenko *et al.* [27].

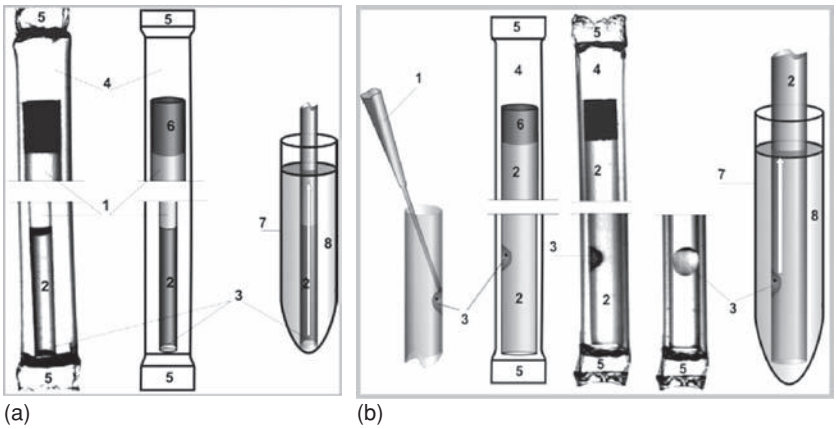


**Figure 14.9** Two modifications of aseptic vitrification technique for spermatozoa. (a) Photograph of container and method for "Open Pulled Straw" vitrification and warming of spermatozoa. (1) Open pulled straw. (2) Suspension of spermatozoa (5–10 μl). (3) Meniscus of suspension. (4) 90 mm straw. (5) Heat-sealed end of 90 mm straw. (6) Marked end of Open Pulled Straw. (7) Tube for warming. (8) Warming medium. White arrow indicates the direction of thawing and swim-up of sperm suspension. (b) Photograph of container and method for open straw vitrification and warming of spermatozoa. (1) Tip of pipette. (2) Open straw. (3) Drop of spermatozoa (0.5–2 μl). (4) A 90 mm straw. (5) Heat-sealed end of a 90 mm straw. (6) Marked end of open straw. (7) Tube for warming. (8) Warming medium. White arrow indicates the direction of thawing and swim-up of sperm suspension. With permission from Isachenko *et al.* [49].

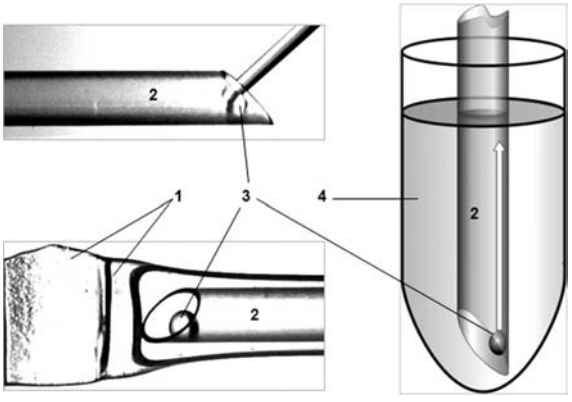

**Figure 14.10** Photographs and scheme of warming using the Cut Standard Straw (CSS) container for vitrification. (1) A closed 0.5 ml straw. (2) Cut Standard Straw. (3) Vitrification medium with embryo. (4) Tube with solution for warming and removal of cryoprotectant. With permission from Isachenko *et al.* [77].

sealed from both sides with a hand-held sealer (Medical Technology GmbH, Bruckberg, Germany) and plunged into $LN_2$. The warming of the spermatozoa sample was performed a day earlier than the planned intracytoplasmic sperm injection procedure as follows: the package system was partly removed from $LN_2$ and the over part of the 0.5 ml straw was cut off. Then the CSS was removed from the packaged straw and quickly immersed into 1.5 ml, pre-warmed to 37°C, HTF with 1% HSA in a 2 ml Eppendorf tube. This mode allows very quick warming, with a speed of approximately 90 000°C/min. Next, the spermatozoa were concentrated by centrifugation at 340 g for 3 min. The resulting pellet was resuspended in 10 μl of the same medium and used for further culture, evaluation and ICSI. The motility of spermatozoa was estimated with a Makler's chamber under the light microscope at × 400 magnification. At the same time the following parameters were assessed: progressive motility (categories "a" and "b"); viable, acrosome-reacted, capacitated spermatozoa; and stability of mitochondrial membrane (M $\Delta\Psi$). After 30 min post-warming, the spermatozoa showed the following rate of physiological–morphological parameters: 85% motility (a + b); 10% with signs of capacitation; 5% with signs of acrosome reaction; and 70% spermatozoa with non-damaged mitochondrial membrane.

For induction of superovulation, pituitary suppression was achieved using a long protocol with a gonadotropin-releasing hormone (GnRH) analogue microdose, followed by ovarian stimulation with

recombinant follicle stimulating hormone (FSH) and human menopausal gonadotrophin.

Ten cumulus-oocyte complexes were retrieved 36 h after the administration of 10 000 IU of human chorionic gonadotropin (hCG). The oocytes were denuded with 80 IU/ml of hyaluronidase and 8 mature MII oocytes were microinjected with vitrified/warmed spermatozoa. Eighteen hours later, six oocytes showed signs of normal fertilization. The embryos were cultured in vitro for 48 h and transferred to the uterus cavity (Frydman Ultra Soft Catheter with Echo Tip, CCD, France) under ultrasonographic guidance.

Fifteen days after transfer, a β-hCG value of 148 IU/L was registered, a clinical twin pregnancy was confirmed by ultrasound at 7 weeks of gestation and 2 healthy babies were born at term.

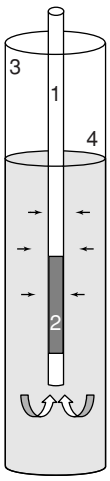


**Figure 14.11** The scheme of the warming process of a vitrified spermatozoa sample. (1) A sample straw. (2) A spermatozoa sample. (3) A 15 ml plastic centrifuge tube. (4) Sperm preparation medium. The arrow heads shows the direction of the heat moving from the warm sperm preparation medium through the plastic wall of straw to the vitrified spermatozoa sample. The large arrows below the sample straw shows the direction of movement of the warm sperm-preparation medium inside of the sample straw. The direct contact between the warm sperm preparation medium and the vitrified spermatozoa sample accelerates its melting.

## Is vitrification suitable for IVF, ICSI and insemination?

Compared to conventional slow freezing, all modifications of the vitrification technique only allow the simultaneous cryopreservation of a small amount of spermatozoa suspension (from 0.5 to 30.0 μl). So, the advantage of programmable or non-programmable conventional slow freezing [17–19] is the ability to simultaneously preserve a relatively large volume of diluted ejaculate or prepared spermatozoa (from 0.25 to 1.0 ml) [20–22, 50]. Because of this, our subsequent study aimed to develop an aseptic vitrification technique for handling a large volume of spermatozoa suspension, which would allow this method to be applied for purposes such IVF or intrauterine insemination as they require a large volume of spermatozoa.

We used the results of our previous investigation concerning slow vitrification and a quick warming rate [27] to help us develop an aseptic vitrification technique for handling large volumes of spermatozoa suspension [128]. This is because the packaging system, when compared with the sample directly making contact with $LN_2$, considerably slowed down the speed of cooling. This problem was solved by using for the warming step a large volume of warm (37°C) sperm preparation medium (5 ml) with gentle agitation to accelerate the melting, and the removing of unclosed content from both side straws for the sample. Such a system allows the transition of heat from the warm sperm preparation medium to the sample through the plastic wall and, at the same time, provides direct contact between warm solution and the solid sample (Figure 14.11). Such straw's filling and warming achieves the necessary speed of warming and protection required for spermatozoa's life-important parameters. To check the before and after results of vitrification/freezing, we analyzed spermatozoa motility and applied highly sensitive methods to analyze the cells' integrity and function.

Flow cytometry (FACSCalibur, Becton Dickinson) was applied to analyze the following:

1 *Mitochondrial membrane potential integrity*. To evaluate mitochondrial activity, we measured the changes in the $M\Delta\Psi$ using a unique fluorescent cationic dye, 5,5′, 6,6′-tetachloro-1–1′, 3,3′-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1 [118].
2 *Cytoplasmic membrane integrity (CMI)*. The integrity of the plasma membrane was assessed with a LIVE/DEAD sperm viability kit, which is used to stain nucleic acid probe molecular (SYBR-14 dye) and propidium iodide (IP).
3 *Acrosomal membrane integrity (AMI)*. The acrosomal membrane integrity was assessed using the technique described by Mendoza *et al*. [129].
4 *Phosphatidylserine translocation (PST)*. To determine of the phosphatidylserine translocation in the sperm, we applied the anexin V-FITC staining technique (APOPTEST$^{TM}$-FITC, Nexins Research, the Netherlands).
5 *Level of DNA fragmentation*. For determination of DNA fragmentation in spermatozoa [130], we used TUNEL (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science, USA), which

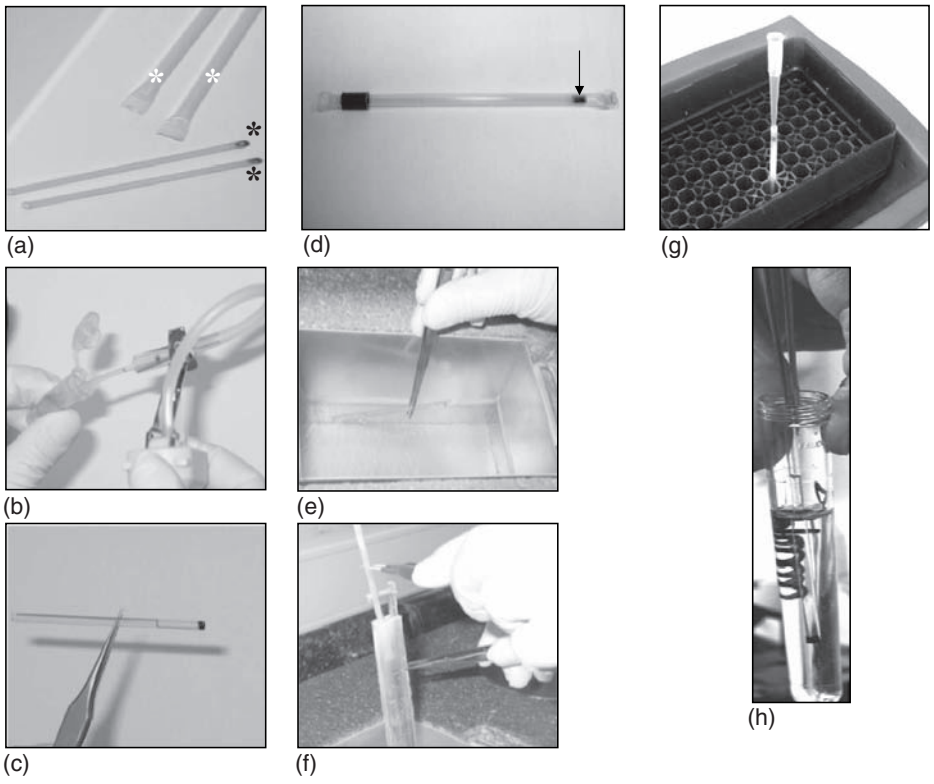


**Figure 14.12** The vitrification procedure for big volume of spermatozoa suspension. (a) The 0.25 ml plastic straws are halved and dark-marked from one side (dark asterisks) and a 0.5 ml packaging straw (white asterisks). (b) The non-marked side of the half-straw is filled with spermatozoa suspension. (c) A 0.25 ml half-straw, hermetically closed from both sides, is filled with spermatozoa suspension. (d) Holding it in a horizontal position, the 0.25 ml half-straw filled with spermatozoa suspension is marked from one side (arrowed) and placed into a 0.5 ml packaging straw, closed from both sides. (e) Using tweezers and keeping it in a horizontal position, this closed packaging system is directly immersed into liquid nitrogen ($LN_2$) and submerged for over 5 s to prevent the flow of spermatozoa suspension spreading into packaging straw. (f) The vitrified sample is stored in $LN_2$. Using tweezers, the dark-marked part of the Sealed Pulled Straw (SPS), approximately 1.0–1.5 cm, is removed from the $LN_2$ and the end of packaging straw is cut. (g) With the help of a 200 μl pipette tip (Eppendorf AG, Hamburg, Germany), the suspension-filled fixed straw is quickly removed from the packaged straw and (h) immersed into a 15 ml plastic tube containing 6 ml of human tubal fluid (HTF) and human serum albumin (HSA) prewarmed to 37°C with gentle agitation to accelerate the melting and removing of content. See plate section for color version.

is based on the detection of single and double-stranded DNA breaks occurring at early stages in apoptosis.

The results of this technique were compared to the slow conventional frozen technique. For this purpose all supernatants from each swim-up-prepared ejaculate were centrifuged, then diluted with the same medium to achieve the concentration of $5 \times 10^6$ spermatozoa/ml and then finally divided into three equal parts. The first part non-frozen swim-up prepared spermatozoa served as a control, the second part was conventionally frozen and third part was vitrified.

Conventional freezing/thawing was performed according to routine procedure in $LN_2$ vapor (–80°C 10 cm over the $LN_2$ surface) for 30 min using freezing medium. The frozen samples were stored a minimum of 24 h before evaluation. The thawing of samples was performed in a 37°C water bath, washed for 5 min at 340 g and pellets resuspended with HTF–HSA.

The vitrification/warming method [128] for a relatively large volume of spermatozoa suspension (100 μl, compared to small volume ~1–30 μl) was performed as follows (Figure 14.12a–f): The suspension of swim-up-prepared spermatozoa was diluted 1:1 with a solution of 0.5 M sucrose (0.25 M end concentration) to

achieve the final concentration of $5 \times 10^6$ spermatozoa/ml. For vitrification, one half of the 0.25 ml plastic straw was used. For this, the straw was cut into two parts and a dark mark made from one side. All subsequent manipulations were done strictly in a horizontal position to prevent the loss of spermatozoa suspension. The half-straw was filled from the non-marked side with 100 μl of spermatozoa suspension. Then sticky tape in a horizontal position was put into a 0.5 ml packaging straw, previously hermetically closed from the other side, and hermetically closed. Then the straw packaging system (SPS), held by tweezers to strictly keep it in a horizontal position, was directly immersed into $LN_2$ and submerged there for over 5 s to prevent the flow of spermatozoa suspension into the packaging straw. It was then stored in $LN_2$ for at least 24 h before use.

The warming of spermatozoa suspension was done as follows (Figure 14.12g,h): Before removing the SPS from the $LN_2$ it was necessary to find the side of the dark-marked spermatozoa suspension-filled straw. Using tweezers, this part of the SPS, approximately 1.0–1.5 cm, was removed from the $LN_2$. The end of the packaging straw was cut and then, with the help of pipette tip for 200 μl, the suspension-filled straw was fixed, quickly removed from the packaged straw and immersed into a 15 ml plastic tube containing 10 ml of HTF–HSA prewarmed to 37°C with gentle agitation to accelerate the melting and removing of content. After warming, the spermatozoa were concentrated by centrifugation at 340 g for 5 min and the sediment was resuspended with HTF–HSA. As a control, before cryopreservation we tested the influence of basic medium (HTF–HSA), vitrification medium (HTF–HSA–sucrose) and the TEST–yolk–buffer (TYB) medium used for conventional freezing, on the physiologic parameters of spermatazoa (Figures 14.13, 14.14). The motility of spermatozoa 1 h after warming, with subsequent incubation at 37°C in a $CO_2$ atmosphere, and after 24 h in vitro culture is shown in Figure 14.13. The data shows that our newly developed method of vitrification for relatively large volumes of spermatozoa suspension (100 μl, compared to small volume ~1–30 μl) achieves high spermatozoa motility (62%) after warming and satisfactory motility after 24 h in vitro culture (22%). This is not statistically different from slow frozen spermatozoa at 24 h (24%). Indeed, all of the data are similar to the slow-freezing technique.

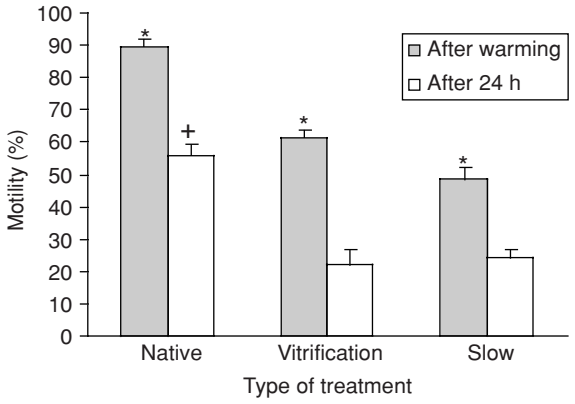


**Figure 14.13** Influence of type of sperm treatment on spermatozoa motility after warming/thawing and after 24 h of in vitro culture. Different superscripts indicate significant differences ($P < 0.05$).

The influence of the type of treatment on the stability of mitochondrial membrane potential M $\Delta\Psi$, the plasmatic and acrosomal membrane, induction of cryo-capacitation (phosphatidylserine translocation) and level of DNA fragmentation is shown in Figure 14.14. The data shows that aseptic vitrification had a significantly stronger protective effect on the mitochondrial membrane's (Figure 14.14a) potential stability (72%) compared to conventionally frozen spermatozoa (30% $P < 0.05$).

The integrity of the cytoplasmic membrane (Figure 14.14b) was significantly affected by both cryopreservation methods, but protection of the cytoplasmic membrane by conventional slow freezing was significantly lower (30%, $P < 0.05$) than by vitrification (54%).

Both aseptic vitrification and conventional freezing negatively influence the acrosomal membrane integrity (28.0 ± 6.9% versus 41.4 ± 2.5%, respectively; $P < 0.05$; Figure 14.14c). Although the acrosomal membrane integrity was lower after applying aseptic vitrification, the index of spermatozoa integrity was non-statistically different from the number of conventionally frozen spermatozoa with non-damaged acrosomal membrane.

It was noted that this vitrification technique strongly prevented the translocation of phosphatidylserine (2%; Figure 14.14d) compared to phosphatidylserine quantitative indexes of conventionally frozen spermatozoa (20%; $P < 0.01$). Both

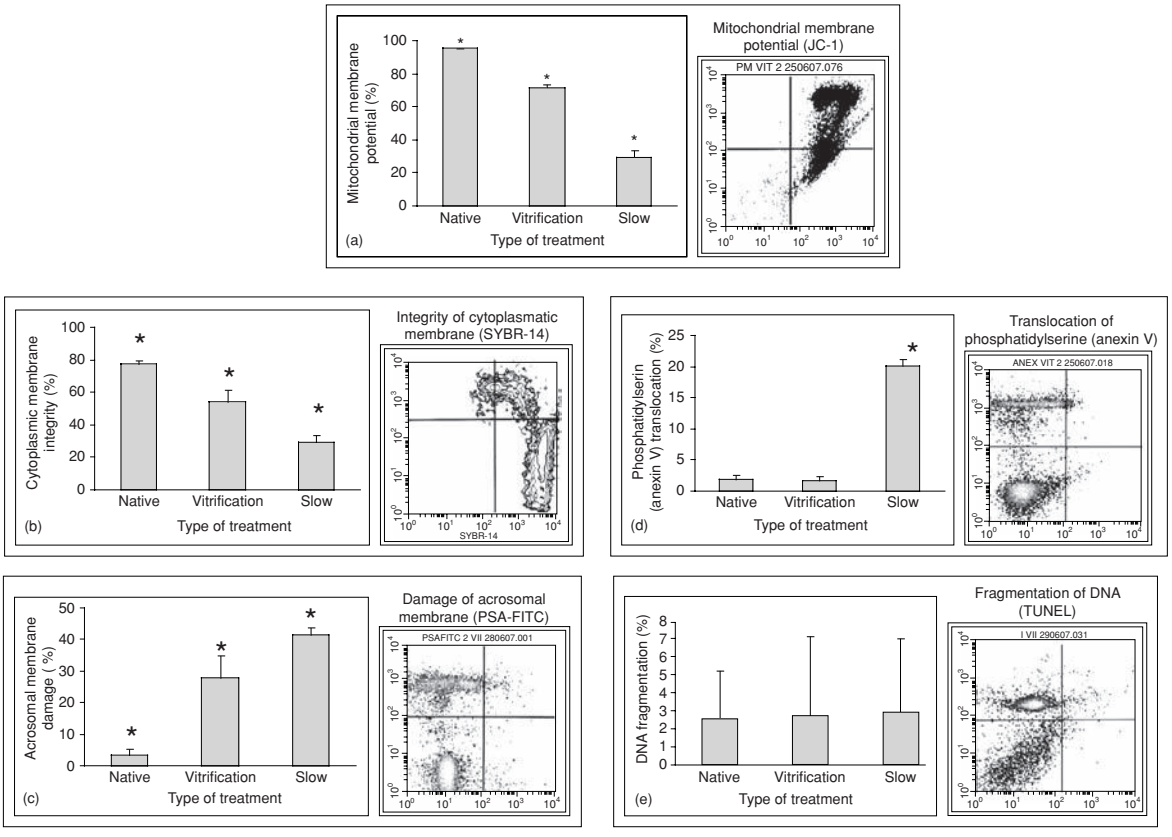


**Figure 14.14** Influence of type of sperm treatment on spermatozoa mitochondrial membrane potential integrity, plasmatic and acrosomal membrane integrity, stability of phosphatidylserine and level of DNA fragmentation. (a) Mitochondrial membrane potential integrity. (b) Plasmatic membrane integrity. (c) Acrosomal membrane integrity. (d) Phosphatidylserine (anexin-V) translocation ability. (e) Level of DNA fragmentation. Different superscripts indicate significant differences ($P < 0.05$).

methods of cryopreservation, aseptic vitrification and conventional freezing (Figure 14.14e) had no negative influence on cells' DNA integrity ($P > 0.5$).

## Future perspectives on the long-term storage of male gametes

Long-term storage of frozen cells and tissues remains elusive in both theoretical and routine cryobiology, and future investigation applying nanotechnology is needed. The principle behind future investigations is that the developmental rate of cells depends on the concentration of cryoprotectants and the speed of cooling and warming. Right now, cryobiology investigations are exploring vitrification and freezing-drying (lyophilization) techniques.

Vitrification is a future perspective in the long-term storage of male gametes because the method is cheap, quick and successful for different types of reproductive (different stages of maturity), somatic, stem cells and, seemingly, tissues. Due to the lowering of a solution's glass transition temperature, permeable cryoprotectants:

- prevents actual freezing and
- maintains some flexibility in a glassy phase.

Vitrification without permeable cryoprotectants also allows us to:

- avoid cryoprotectants toxicity and osmotic stress
- avoid damage to the plasmatic and mitochondrial membrane during equilibration with cryoprotectants
- protect plasmatic and mitochondrial membrane against lipid peroxidation and the formation of reactive oxygen species
- avoid DNA damage.

The other future perspective in the long-term storage of male gametes is lyophilization. This method provides a low cost for storage and transport of preserved materials because the biological material can be stored at 4°C and shipped at ambient temperatures. This technique is very suitable for preservation of the genome (nucleus), because nuclear viability is not equivalent to cell viability and is not destroyed by freezing/drying [131–135]. In 2003, Ward, from the Yanagimachi group, wrote wrote that: (1) the freeze-drying procedure itself causes some abnormalities in spermatozoa but freezing without cryoprotection does not; and (2) long-term storage of both frozen and freeze-dried spermatozoa is not deleterious to their genetic integrity [136]. Freezing without cryoprotection is highly successful, simple and efficient but, like all routine sperm storage methods, requires $LN_2$. Liquid nitrogen is also required for freeze-drying but sperm can then be stored at 4°C and shipped at ambient temperatures. Both preservation methods are successful, but rapid freezing without cryoprotection is the preferred method for preservation of spermatozoa from mouse strains carrying unique genes and mutations. The genomic integrity of cells can be maintained after freeze-drying and it is possible to produce offspring from the cells using nuclear transfer techniques [131, 137, 138]. In this case, the long-term preservation of mouse sperm by desiccation is economically and logistically attractive [139].

## Conclusions

- It is feasible for human spermatozoa to be vitrified without using permeable cryoprotectants. This is either by directly plunging into $LN_2$ or freezing in $N_2$ vapor beforehand, followed in both cases by rapid thawing.
- The speed of warming plays a decisive role in vitrification independent from the type of cells.
- The DNA of spermatozoa from normospermic samples is stable and independent from the type of treatment.
- A mixture of non-permeable cryoprotectants (HSA + sucrose) can significantly enhance mitochondrial integrity and prevent initiation of capacitation and the acrosome reaction process.
- The vitrification procedure without permeable cryoprotectants effectively protects the important physiological parameters of spermatozoa.
- The developed aseptic vitrification technique for large volume (100 μl) spermatozoa suspension retains the full functionality of spermatozoa.
- In contrast to conventional freezing, the method is quick and simple and does not require special cryobiological equipment.
- The evaluation of motility and long-term survival of spermatozoa allows us to conclude that all four investigated methods can be used successfully. However, the method of aseptic vitrification is recommended because it minimizes the potential risk of microbial contamination.

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Chapter 15

# Transplantation of cryopreserved spermatogonia

Jill P. Ginsberg and Ralph L. Brinster

## Background

Over the last several decades, survival rates for childhood cancer have steadily increased. In fact, with the overall cure rate for pediatric malignancies now approaching 80%, current estimates indicate that 1 in every 640 young adults in the USA will be a survivor of childhood cancer [1]. Unfortunately, many survivors struggle with medical late effects of their treatment including disorders of the endocrine system, cardiac and pulmonary dysfunction, secondary neoplasms and infertility. Gonadal damage is a relatively common consequence of the treatments used to cure pediatric cancer. The extent of cytotoxic germ cell damage depends on the specific chemotherapeutic agents used and the cumulative doses received. Alkylating agents (particularly cyclophosphamide, ifosfamide, nitrosureas, chlorambucil, melphalan, busulfan and procarbazine) are the most common class of drugs known to effect gonadal function and their impact has been studied extensively [2]. Additionally, the testes have a very low threshold for radiation exposure, and even small doses are known to be gonadotoxic. As treatment regimens for pediatric oncological malignancies have improved, more and more survivors are entering their reproductive years [3]. Maintenance of fertility is extremely important with regard to long-term quality of life for these survivors [4, 5]. Consideration must be given to whether a child's fertility is likely to be impacted by his treatment. Ideally, this should occur before the start of therapy, when a window of opportunity may exist to preserve the patient's future reproductive potential [4–8]. Pubertal males can produce a semen sample prior to starting gonadotoxic therapy and cryopreserve the sperm for future use. Because current methods for oocyte fertilization can utilize as few as one motile sperm (e.g. intracytoplasmic sperm injection [ICSI]), this method has proven to be successful even when the number of cryopreserved sperm is small [5, 9, 10].

Unfortunately, pre-pubertal males pose a particular challenge for fertility preservation because these boys cannot produce mature spermatozoa for cryopreservation. During embryonic development, primordial germ cells (PGCs) migrate to the genital ridge and differentiate into gonocytes [11, 12]. In the mammalian postnatal testis, gonocytes are the first cell population committed to male germline development. Before puberty, these cells then give rise directly to spermatogonial stem cells (SSCs). In the mouse, which has a short pre-pubertal period (~3 weeks), some gonocytes transition to SSCs and others undergo an early differentiation directly to type A1 spermatogonia by day 6 of life. During the first 2–3 months after birth in humans, which have a long pre-pubertal period (~12 years), the gonocytes are replaced by adult dark (Ad) and adult pale (Ap) spermatogonia that are thought to represent the reserve SSC and active SSC pool [12, 13]. These Ad and Ap spermatogonia undergo activation beginning at approximately age 5 years, particularly to type B spermatogonia. By age 10 years, these SSC represent about 10% of total spermatogonia. During puberty, the SSCs in all species provide the foundation for spermatogenesis, through self-renewal and differentiation to daughter cells. Although the germ cells of the pre-pubertal testis contain a small number of the self-renewing SSCs they do not yet have mature spermatozoa. For these at risk pre-pubertal boys, current practice does not provide any options for fertility preservation at cancer diagnosis.

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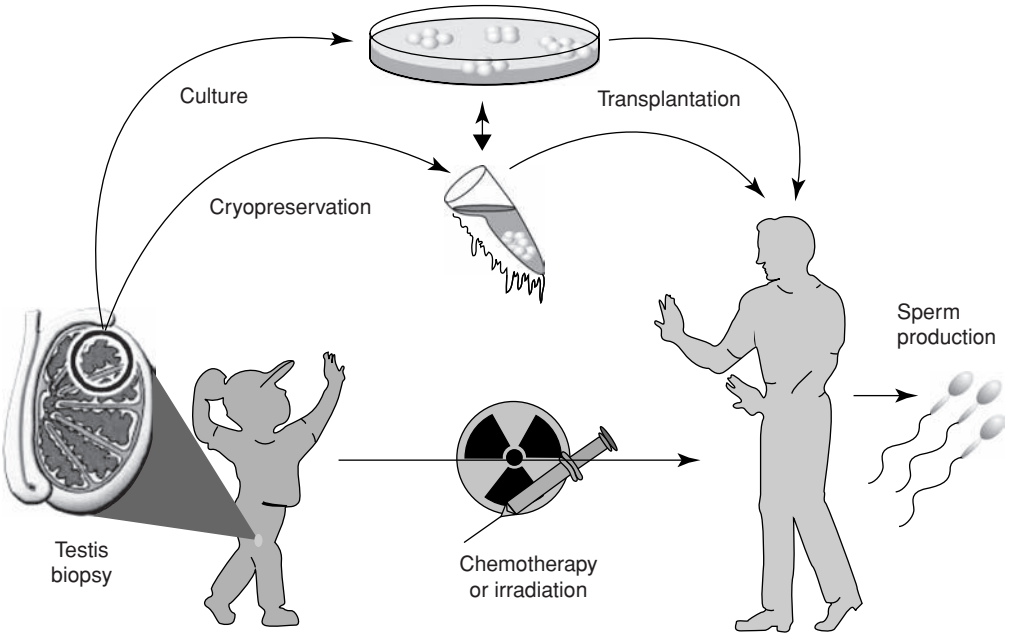



**Figure 15.1** Male germline stem cell preservation. Before treatment for cancer by chemotherapy or irradiation, a boy could undergo a testicular biopsy to recover stem cells. The stem cells could be cryopreserved or, after development of the necessary techniques, could be cultured. After treatment, the stem cells would be transplanted to the patient's testes for the production of spermatozoa. From Brinster [14] with permission. See plate section for color version.

A potential approach to this issue is the use of cryopreserved testicular tissue. Ideally, pre-pubertal testicular tissue could be acquired and banked prior to initiating gonadotoxic cancer therapy (Figure 15.1, [14]). Years later, once the patient is ready to begin a family, this tissue could then be thawed and the stored germ cells re-implanted into the patient's own testes [14, 15]. Alternatively, the stored cells could be matured and expanded in vitro until they can achieve fertilization by use of ICSI. Cryopreservation of the testicular tissue has been shown to be feasible in many species including mouse, rat, pig, baboon and humans, and the ability to of restore spermatogenesis are equivalent to using fresh cells [11, 16, 17]. Unfortunately, the initial testicular biopsy, which is small, contains very few SSCs, and cryopreservation and techniques used to remove malignant cells results in loss of SSCs. Therefore, there is an insufficient number to result in fertility following transplantation. For this transplantation approach to be useful clinically, stem cells from the biopsy sample must be isolated and expanded in vitro prior to re-introduction [15]. While significant strides have been made in animal model research in this area, translational use of testicular tissue cryopreservation in humans remains experimental [9, 18].

## Development of spermatogonial transplantation

Spermatogonial stem cells are responsible for the continual production of spermatozoa throughout adult life. Both SSCs and the surrounding cells in the seminiferous tubules regulate the biological activity of these cells. Considerable research has been dedicated to understanding the interactions between SSCs and the surrounding somatic cells for proper sperm production. For example, Sertoli cells are thought to be extremely important for SSC growth and development by secreting growth factors that regulate these germ cells [19, 20]. Importantly, a critical breakthrough in the characterization of SSCs has been the development of the germ cell transplantation technique [11, 15, 21]. In 1994, Brinster and Avarbock developed the first animal model of SSC transplantation [21]. Injection of spermatogenic cells into the seminiferous tubules gave rise to donor cell-derived foci of spermatogenesis in the recipient testes.

Figure 15.2 presents a schematic overview of spermatogonial transplantation in mice. First, transgenic mice carrying a LacZ or green fluorescent protein (GFP) transgene are used as donor mice. The marked

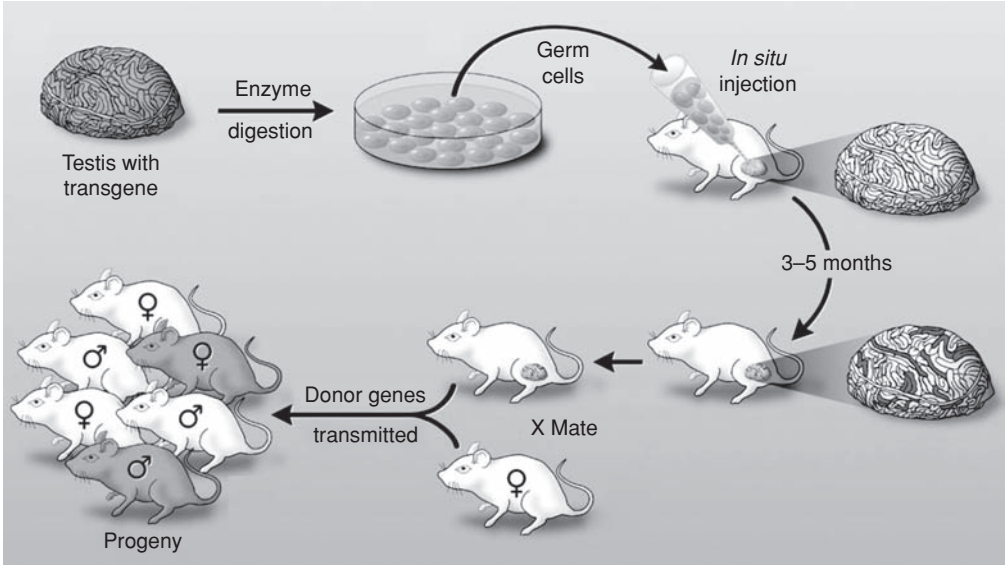



**Figure 15.2** Testis cell transplantation method. A single-cell suspension is produced from a fertile donor testis. The cells can be cultured or microinjected into the lumen of seminiferous tubules of an infertile mouse. Only a spermatogonial stem cell can generate a colony of spermatogenesis in the recipient testis. When testis cells carry a reporter transgene that allows the cells to be stained blue, colonies of donor cell-derived spermatogenesis are identified easily in the recipient testes as blue stretches of tubule. Mating the recipient male to a wild-typed female produces progeny, which carry donor genes. From Brinster [11] with permission. See plate section for color version.

donor testis cells are digested to make a single-cell population suspension. The cell suspension is introduced into the seminiferous tubules of recipient mice testis that contain few to no germ cells after treatment with busulfan that destroys endogenous spermatogenesis. The recipient mice can become fertile to father progeny. Notably, resultant offspring carry the donor mouse haplotype [21]. Research utilizing animal models of male infertility (e.g. busulfan-treated nude mice) has demonstrated that there are several methods to use germ cells from testicular tissue to obtain mature spermatozoa for fertilization, including autotransplantation, allotransplantation and xenotransplantation [11, 22]. Autotransplantation is considered more acceptable than allotransplantation or xenotransplantation, although both of the latter have been used successfully in mouse models [17, 21].

## Morphological identification of spermatogonial stem cells

The number of SSCs is very low in the testis of an adult mouse. It is estimated that SSCs constitute only about 0.03% of testicular germ cells [23]. Typically, about $10^6$ germ cells are introduced into the recipient testis depending upon stem cell concentration, among which only a few hundred cells could be stem cells. About 20 spermatogenic colonies will develop, depending on the stem cell concentration. Colonization efficiency is estimated to be about 5–10%.

The paucity of SSCs in comparison to differentiating germ cells and somatic cells within the testis has challenged the field to develop reliable markers of these specialized germ cells, so they can be identified unequivocally for subsequent isolation and enrichment in vivo or in vitro. Tangible advances include identification of SSCs or SSC-like spermatogonia by morphology, chemical phenotype and functional characteristics in relevant animal models. For example, the study of rodent SSCs was previously hampered by the lack of techniques for purification and long-term in vitro maintenance. However, recent methodological advances have been developed and refined for rodent germ cell identification and transplantation along with improved culture conditions for SSC expansion and growth [20, 24–26]. These techniques have led to

the characterization of many aspects of SSC biology, including the identification of growth factors such as glial cell line-derived neurotrophic factor (GDNF) as the main regulator of rodent SSC self-renewal [25–27]. Morphologically, gonocytes that give rise to SSCs are larger in diameter than nearby somatic cells, and tend to rest loosely on the basement membrane of the seminiferous tubules. Because of the difference in size and morphological characteristics between gonocytes and somatic cells and absence of the differentiating stem cells, micromanipulation techniques to select these two cell types from single cell suspensions isolated from pre-pubertal human and mouse testes have been enabled [27]. Selected populations were distinctly homogenous, and virtually pure populations of germ cells and somatic cells have been obtained and validated phenotypically by immunological techniques for well-established markers that differentiate gonocytes/SSCs from somatic cells [27]. For example, selected gonocytes from mouse testes and spermatogonia from human testes have been identified using the markers zinc finger and BTB domain containing 16 (ZBTB16), ubiquitin carboxyl-terminal esterase L1 (UCHL1) and deleted in azoospermia-like (DAZL), along with a lack of labeling for GATA-binding protein 4 (GATA4), which is found in Sertoli cells but not germ cells [19, 28, 29]. In contrast, selected populations of somatic cells were negative for ZBTB16, UCHL1 and DAZL and positive for GATA4. Data indicate that micromanipulator selection of gonocytes from pre-pubertal human testis and neonatal mouse testis cell suspensions is an effective technique for the enrichment of germ cells and provides essentially pure ($\geq$ 99%) populations of cells for downstream molecular and cellular analyses as well as germ cells for transplantation purposes. These advances also enable future comparisons of SSCs from humans and rodent species for planned transplantation interventions. Moreover, a similarity in self-renewal and survival mechanisms between human and mouse SSCs may exist, because transplantation of testis cells from non-rodent species, including human, into testes of immunodeficient mice allowed the maintenance and limited replication of spermatogonia in the recipient seminiferous tubules for periods of 6–12 months [11, 17, 27]. Additionally, comparison of molecular and cellular fingerprints from isolated human spermatogonia and mouse gonocytes could provide details regarding specific gene expression patterns. The degree and characteristics of gene expression similarity would allow extrapolation of our knowledge about mouse SSCs to the difficult study of human germline cells, and ultimately impact our understanding of human male fertility and infertility.

## Isolation, purification and culture of murine spermatogonial stem cells

As stated above, the number of SSCs in the male mouse testes is relatively low and identification of these cells is not straightforward. In 1999, Shinohara *et al.* demonstrated that $\beta_1$ and $\alpha_6$-integrins are specific surface markers for mouse SSCs [30]. Cells that were positive for these markers were selected using a magnetic bead procedure on a testicular cell suspension. Using this method with the transplantation model, there were a greater number of colonies of spermatogenic cells originating from donor cells in the recipient when enhancement of the concentration of the SSCs was used [31]. In 2004, Kubota *et al.* demonstrated the essential role of glial cell line-derived neurotrophic factor (GDNF) for in vitro proliferation of SSCs, which were enriched from mouse testes [25]. Using a well-established enrichment strategy, (Thy-$1^+$) SSCs were identified and isolated. The stem cells were then cultured in a well-defined serum-free medium, which led to successful expansion, and enabled identification of essential growth factors for this critical cell type. Importantly, Kubota *et al.* demonstrated that these stem cells grew best in culture with the addition of specific growth factors and their cognate receptors, including GDNF, basic fibroblast growth factor (bFGF), and GDNF family receptor alpha-1 (GFR$\alpha$1) [25]. They cultured murine SSCs for 4 months, and then in vivo spermatogenesis was restored after transplantation back into the recipient [25].

Over the last decade, methods have been developed for rodent germ cell transplantation and SSC culture conditions [11, 20, 24–26]. Specifically, GDNF has been established as the main regulator of rodent SSC self-renewal [25, 26]. The c-Ret receptor tyrosine kinase (RET) and the cofactor GFR$\alpha$1 bind to initiate intracellular signaling cascades within SSCs [25, 27, 32]. By examining GDNF withdrawal in rodent SSC cultures, several GDNF-dependent genes have been identified, including B-cell CLL/lymphoma 6, member B (Bcl6b), basic helix-loop-helix family, member e 40 (Bhlhb2), Ets variant gene 5 (Etv5),

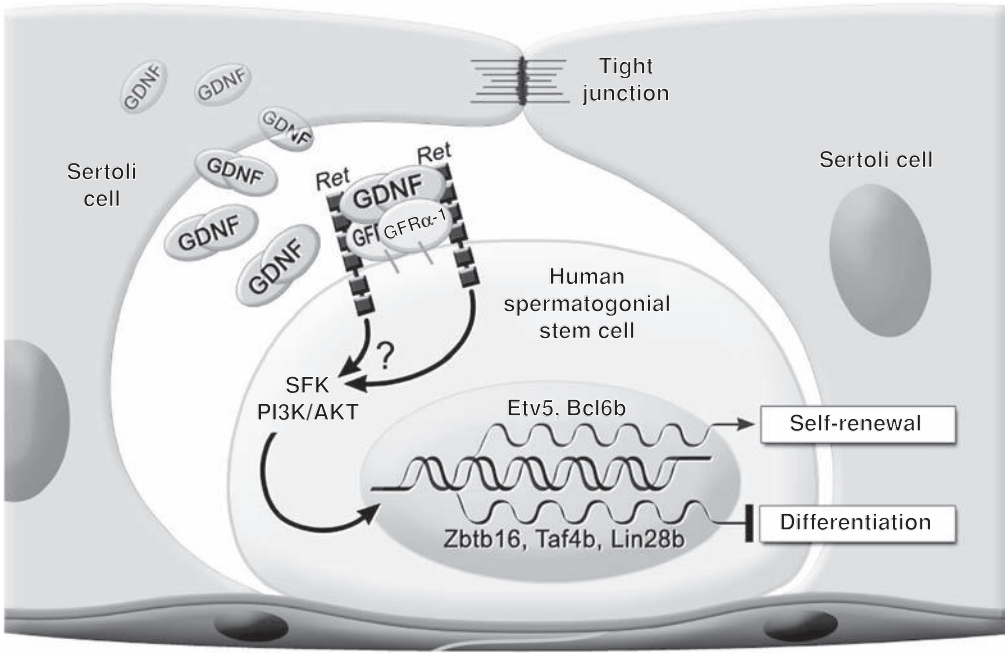



**Figure 15.3** A proposed model of human spermatogonial stem cell (SSC) self-renewal regulation by glial cell line-derived neurotrophic factor (GDNF), which has been demonstrated to have an essential role in regulating rodent SSC self-renewal. The model is similar to those suggested for mouse SSC self-renewal. In this model, GDNF binds to RET and the GFRα1 coreceptor with possible intracellular protein kinase signaling through SFK and PI3K/AKT downstream pathways to regulate the expression of specific genes, such as Etv5 and Bcl6b, which are involved in SSC self-renewal. However, other genes not regulated by GDNF (e.g. Zbtb16, Taf4b and Lin28), are likely controlled by different signals and may block differentiation but not be involved directly in self-renewal. Genes for these regulatory molecules have been shown to be highly expressed in pre-pubertal human spermatogonia, mouse gonocytes and mouse SSCs. The basement membrane (green), on which the SSC rests, is generated by the peritubular myoid cells (dark brown) and Sertoli cells (tan). From Wu *et al.* [27] with permission. See plate section for color version.

homeobox C4 (Hoxc4), LIM homeobox 1 (Lhx1) and Tec protein tyrosine kinase (Tec) [27, 32, 33]. Notably, Bcl6b and Etv5 have implicated by several independent research studies to be involved in regulating rodent SSC self-renewal [32–36]. Glial cell line-derived neurotrophic factor also activates downstream signaling cascades including phosphatidylinositol 3-kinase (PI3K), serine-threonine kinase AKT family (AKT) and Src family kinase (SFK) that impact rodent SSC maintenance and self-renewal (Figure 15.3). Thus, GDNF is considered a factor critical for SSC self-renewal. Importantly, inclusion of GDNF in culture media is essential for SSC self-renewal in vitro and additional supplementation with bFGF or spidermal growth factor (EGF) augments those effects. Currently, SSC culture systems that support long-term SSC self-renewal are available only for mouse, rat and hamster [34]. Additional research is required to determine which specific cell surface and/or intracellular markers are expressed on human testicular SSCs to enable the rapid and reproducible accession of enriched SSC cell populations for downstream analyses and clinical utilization.

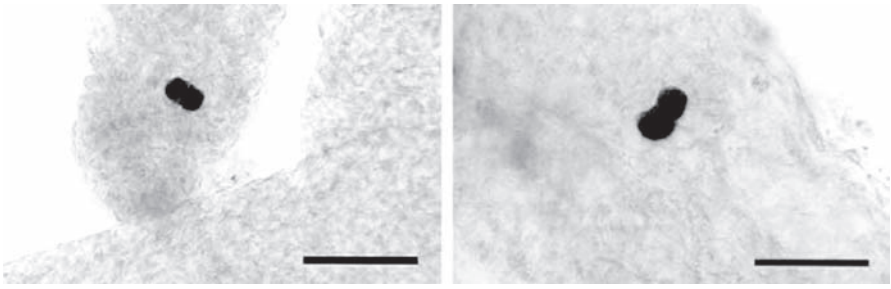
## Culture of human spermatogonial stem cells

Spermatogenesis in vitro from biopsied germ cells is considered to be an excellent alternative for pre-pubertal boys with malignancies, particularly of hematopoietic origin, who carry a risk of relapse after transplantation. The ability to mature stem cells to spermatids in vitro would offer an important option to pre-pubertal cancer patients. Unfortunately, enormous hurdles remain for bringing the in-vitro maturation processes into the clinical setting. Even for autotransplantation of SSCs a major challenge is that human germ cells, similar to murine germ cells

described above, likely yield a low number of SSCs, as $10^4$ germ cells may contain only 2 or 3 stem cells [14, 23]. Methods are needed to isolate and increase the number of human SSCs available to be subsequently autotransplanted or matured in vitro. Unfortunately, the initial biopsy contains very few SSCs, and success of either of these procedures is likely to be equivocal. Therefore, even for autotransplantation, which is a procedure already established in animals, to be used clinically, stem cells from the biopsy sample must be isolated and expanded in vitro prior to re-introduction [14]. Spermatogonial stem cell isolation from pre-pubertal human testis biopsy samples by itself is not likely to be sufficient to restore fertility following autologous transplantation because the number of SSCs recovered from a biopsy is small. Our recent study found that testicular biopsies from pre-pubertal boys ($n = 9$; range 2–10 years) weighed $31.5 \pm 3.7$ mg and provided $3.9 \pm 0.6 \times 10^5$ cells per biopsy [27]. The concentration of spermatogonia is predicted to be about 3% of the cell population (estimated to be approximately 11 700 spermatogonia per biopsy), and the number of Ad and Ap spermatogonia with stem cell potential in this population is unknown. For comparative purposes, spermatogenesis restoration to approximately half of the seminiferous tubules of a sterile mutant mouse testis and resultant fertility to approximately half of sterile mice requires transplantation of approximately 150 SSCs per testis. As the adult human testis (about 12 g) is nearly 120 times larger than a mouse testis (about 0.1 g), approximately 18 000 SSCs would need to be transplanted to each human testis for a comparative level of fertility restoration, assuming the same successful response level was obtained to human SSC transplantation [27]. Furthermore, a crucial factor is verifying that the biopsied testis cells do not contain cancerous cells. This process requires use of cell sorting procedures such as fluorescence-activated cell sorting (FACS, and see later), effectively necessitating more SSCs than recovered from a biopsy [27]. Therefore, simple transplantation of the SSC cells harvested from a single biopsy is not likely to be sufficient to restore fertility. The culture and expansion in number of healthy, cancer-free SSCs is essential for effective clinical use of human SSC transplantation to restore fertility. A recent report indicates that expansion of human germ cells in vitro is feasible [37]. However, much work yet will be required to routinely culture and increase the number of human SSCs and assure their quality.

## Cryopreservation of spermatogonial stem cells

Once spermatogonial transplantation systems were established in vivo, it became critical to determine whether SSCs could be cryopreserved for potential fertility preservation in humans that would otherwise be infertile due to their gonadotoxic pediatric cancer treatments. To date, these procedures are primarily experimental, and the current success lies within animal models, not actual human patients. Notably, the process of SSC transplantation in animal models includes a number of steps in the handling of SSCs, and preparing the receiving testis for transplantation. Stem cells must be appropriately isolated and enriched. For practical purposes in human testis banking, SSCs and related germline cells must be cryopreserved. Cryopreservation enables future manipulation and evaluation of SSCs in culture over a period of time to select cells with the desired properties (i.e. non-malignant). Investigators have routinely collected testis cells from many species after enzyme digestion, frozen these cells and stored them in liquid nitrogen [14, 16, 38, 39]. When ready to be utilized, cells are thawed and transplanted into sterile recipients. This procedure has been determined experimentally to be quite effective, as infertile mice that receive cryopreserved SSC transplants develop donor-cell derived spermatogenesis [16]. To date, several groups have demonstrated that SSCs can be cryopreserved and not lose their ability to restore spermatogenesis in animal models [11, 16, 38]. Transplantation of cryopreserved and fresh testis cells from non-rodent species, including human, into testes of immunodeficient mice has been successfully completed and the data demonstrate that cryopreserved donor human spermatogonia colonize mouse testes similar to fresh spermatogonia (Figure 15.4) [17]. Recently, primate testicular cell suspensions were frozen [38] and, after thawing, viability was found in 58% of the cryopreserved cells. Importantly, Keros *et al.* have documented successful freezing protocols using human testicular tissues [39]. Specifically, slow programmed freezing with 5% dimethyl sulfoxide (DMSO) as a cryoprotective agent is efficient in maintaining spermatogonia, Sertoli cells and the stromal compartment from testicular biopsies during the freezing, thawing and subsequent tissue culture procedures required for successful accession and utilization of human clinical samples. It has been estimated that 50–66% of spermatogonial cells can



(a) (b)

**Figure 15.4** Detection of human germ cells transplanted into seminiferous tubules of recipient mouse testes using a baboon testis specific antibody that identifies human spermatogonia. (a) Donor human spermatogonia in mouse tubule 4 months after transplantation of cryopreserved cells. This panel shows that cryopreserved cells also colonize mouse testes as observed with freshly transplanted cells. (b) Donor human spermatogonia in mouse testis 5 months after transplantation. These donor cells were transplanted without cryopreservation. Bar = 100 μm (a) and 40 μm (b). From Nagano *et al.* [17] with permission from Elsevier Science, Inc. © 2002 American Society for Reproductive Medicine. See plate section for color version.

survive after freezing and thawing, making long-term cryopreservation a feasible means for storing viable cells for future fertility [39].

## Removal of malignant cells

Despite significant advances in spermatogonial cell biology and subsequent fertility management, malignant contamination remains one of the main concerns surrounding autologous transplantation. The risk is substantial with hematological cancers, in which the testes can act as sanctuary sites for leukemic cells. Advances in our ability to detect cancer specific chromosomal or molecular abnormalities will be necessary in order to screen gonadal tissue for malignant cells prior to autotransplantation [40, 41]. Using minimal residual disease detection in hematological diseases might be very useful since one malignant cell can be detected among $10^6$ normal cells [42, 43]. The most sensitive techniques are polymerase chain reaction (PCR) based and use break-point regions of leukemia-specific chromosomal aberrations or the immunoglobulin or T-cell receptor gene rearrangements [43, 44]. Another methodology for screening malignant cells in residual tissues is FACS. Specifically, progress in addressing this question has been made in mouse models, particularly with the use of FACS technology to negatively sort malignant cells from cell suspensions [45–47]. Fujita *et al.* restored fertility in sterile mice after transplanting SSCs isolated from mice with leukemia, without inducing leukemia in the recipient mice [45]. They used positive selection of SSCs by flow cytometry for CD45 (a surface marker for leukemic cells) negative cells. Another alternative method to screen human testicular tissue for malignant potential might be to inject an aliquot of the actual clinical sample suspension into immunodeficient mice prior to delivering into the patient. In this scenario, animal models could be used to test cell suspensions with regard to their malignant potential. If a rodent develops a malignancy following transplantation, then the suspension that was used to inoculate the mouse would be rejected for use in human candidates. Regardless of what method is utilized to remove malignant contamination, the number of SSCs collected from a testicular biopsy need to be further expanded to accommodate for these techniques of removing malignant cells. Indeed, specific cultures of SSCs could be used to eliminate cancer cells that are likely to be dependent on different growth factors and culture conditions.

In summary, the potential for transferring tumor cells within cryopreserved and subsequently cultured and/or expanded testicular tissue back into the patient is of paramount concern. Children most at risk of transmitting cancer cells include those with a hematological malignancy such as acute leukemia. However, local invasion from solid abdominal or pelvic malignancies cannot be excluded such as the patient with rhabdomyosarcoma with testicular involvement. The potential for in vitro maturation of sperm from prepubertal testicular biopsies could overcome this problem by screening and selecting for SSCs that do not have malignant potential.

## Ethical concerns

As testicular cryopreservation is an experimental procedure in humans, efficacy and safety research is governed under the auspices of federal regulations for clinical trials involving children. These federal regulations mandate that federally funded clinical research protocols including children that involve greater than minimal risk, and that present the potential of direct benefit to these subjects, must be reviewed by an Institutional Review Board (IRB) [48]. The IRB approval will only be granted when direct benefits of the experimental treatment outweigh its risks, and that research is likely to provide important information that leads to better understanding of the conditions therein [48]. Strict adherence to these guidelines is crucial regardless of the funding source for clinical trials involving children, as they provide a solid ethical cornerstone for clinical and translational research [48].

Tissue banking centers that accrue gonadal tissue for pediatric cancer patients must inform the parents of the options for disposition of these materials at a future time [49]. These issues are not trivial, as the legal system upholds the prior wishes from an individual regarding the disposition of reproductive material, which is controlling after death. Therefore, tacit instructions that biological materials must be destroyed, or alternatively released to research, have to be honored. Clarity in the handling of biological samples is particularly important in the situation where pre-pubertal patients have cryopreserved gonadal tissue. Specifically, parents or their legal guardians must give directions, in writing in advance, for future tissue disposition, and they must be urged to specify what should be done with banked tissue if their child dies.

As a young child cannot give his consent for the testicular biopsy cryopreservation, parental consent must be granted. Testicular biopsy is an invasive procedure and purely experimental [50]. In practice, a testicular biopsy should be performed under the same general anesthesia that is used to insert a central line for chemotherapy. Although harvesting gonadal tissue may be of high risk if taken in isolation, within the context of the child's illness it may pose minimal additional risk [49]. Therefore, it should be combined with a procedure that requires anesthesia. Lastly, it is important to remember that testicular tissue cryopreservation is still at a highly experimental stage and careful counseling and consent is essential. To be valid, consent must be informed, voluntarily obtained and given by a competent person. The consent process must not raise unrealistic expectations [49, 51].

## Conclusions

Little is known about the biology and regulation of human germline cells, particularly regarding maintenance and regulation of SSCs, which are the foundation of spermatogenesis throughout adult life. Spermatogonial stem cells reside on the basement membrane of the testis seminiferous tubule, and their decision regarding self-renewal versus differentiation determines the efficiency of spermatozoa production. In mice, approximately 0.03% of testes cells are stem cells, and they resemble other early differentiating spermatogonia, a morphological characteristic observed phylogentically across mammalian species [14, 52]. Spermatogonial stem cells arise directly from gonocytes within days in mice and over a few months in humans. The limited knowledge about these critical cells arises from their rarity and relative absence of distinguishing morphological characteristics. To date, cryopreservation is feasible in that all species in which it has been tried; cryopreservation of testicular tissue has been successful with significant numbers of SSCs being recovered. Unfortunately, cryopreservation procedures results in some loss of SSCs and the SSCs from human testicular biopsies are insufficient to re-establish fertility after transplantation. An important goal of future basic and translational research is to generate methods to expand the SSCs in number. In addition to the basic science issues regarding the accession and maturation of SSCs for transplantation, a plethora of practical and ethical issues also need to be addressed for appropriate application of human SSC usage for fertility preservation in boys following gonadotoxic pediatric cancer treatments.

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Chapter 16

# Cryopreservation and transplantation of testicular tissue

Christine Wyns

## Introduction

Due to remarkable advances in the treatment of childhood cancer, we have seen great improvements in life expectancy with up to 80% of children surviving their disease, resulting in a growing population of adult long-term survivors of childhood malignancies [1].

Although oncological treatments are highly effective, a major concern is their adverse impact on fertility [2, 3].

Since rapidly dividing cells are the target of chemo- and radiotherapy, these treatments act not only on cancer cells, but also on germ cells. Differentiating spermatogonia proliferate the most actively and are thus extremely susceptible to cytotoxic agents, although the less active stem cell pool may also be depleted [4].

Consequently, although the pre-pubertal testis does not complete spermatogenesis, there is evidence that cytotoxic treatment given to pre-pubertal boys affects fertility [5]. In addition, the presence of a steady turnover of early germ cells that undergo spontaneous degeneration before the haploid stage is reached [6, 7] may possibly explain why the pre-pubertal state does not offer any protection against gonadotoxic treatments.

Recovery of sperm production after a cytotoxic insult depends on the survival and ability of mitotically quiescent stem spermatogonia (type A *dark*) to transform into actively dividing stem and differentiating spermatogonia (type A *pale*) [8].

The somatic compartment of the testis may be more resistant to chemotherapeutic treatment, since these cells have a low or absent mitotic rate. Nevertheless, increased concentrations of luteinizing hormone (LH) and symptomatic reductions in testosterone levels [2], both signs of Leydig cell impairment, have been described. Evidence of Sertoli cell functional impairment following chemotherapy – responsible for germ cell differentiation inhibition where germ cells have survived – has also been reported [9].

## Loss of fertility: who can benefit from fertility preservation?

Loss of fertility in adult life is a major psychologically traumatic consequence of cancer treatment. Indeed, in a quality of life analysis of former oncological patients, about 80% viewed themselves as potential parents, and the vast majority of younger cancer survivors saw their cancer experience as pivotal in preparing them to be better parents [10]. Therefore, since post-therapy recovery of spermatogenesis remains unpredictable, it is important to inform patients facing infertility as a side effect of their treatment of all the options available to preserve their fertility [3].

There is also considerable evidence that gonadotoxic treatments like hematopoietic stem cell transplantation (HSCT) can cure a variety of non-malignant disorders in children, so fertility preservation should not be reserved solely for boys with cancer [11, 12]. It should also be considered for other benign conditions where seminiferous tubule degeneration is expected over time, such as Klinefelter's syndrome [13]. The indications for immature testicular cryopreservation in case of malignant and non-malignant disease are summarized in Table 16.1.

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
Published by Cambridge University Press. 

**Table 16.1** Indications for immature testicular tissue cryopreservation in case of malignant and non-malignant disease

| Malignant | Non-malignant |
|---|---|
| • Leukemia<br>• Hodgkin's disease<br>• Non-Hodgkin's lymphoma<br>• Myelodysplastic syndromes<br>• Solid tumors<br>• Soft tissue sarcoma | 1 HSCT in case of:<br>• Hematological disorders: thalassemia major, sickle cell disease, aplastic anemia, Fanconi's anemia<br>• Primary immunodeficiencies<br>• Severe autoimmune diseases unresponsive to immunosuppressive therapy: juvenile idiopathic arthritis, juvenile systemic lupus erythematosus, systemic sclerosis, immune cytopenias<br>• Osteopetrosis<br>• Enzyme deficiency disease: Hurler's syndrome |
| | 2 Risk of testicular degeneration<br>• Klinefelter's syndrome |

HSCT, hematopoietic stem stell transplantation.

## Fertility preservation options before gonadotoxic therapies

In order to reduce the deleterious effects of gonadotoxic therapies, different strategies have been tested.

Improving therapeutic regimens using less gonadotoxic protocols [14] could enable spontaneous recovery of spermatogenesis, but their use is not always possible without compromising patient survival.

Limiting radiation exposure by shielding or removing the testes from the radiation field should be implemented whenever possible [3].

Minimizing testicular damage from cancer treatment or protecting SSCs in vivo is another approach. Gonadal protection through hormonal manipulation was reviewed in Chapter 13.

Anti-apoptotic agents such as sphingosine-1-phosphate [15] and AS101 [16] and various other cytoprotective substances [17, 18] have also been used with partial success in rodents.

Currently available drugs to prevent testicular damage from cytotoxic therapy have not proved helpful in humans so far. Testicular tissue cryopreservation therefore remains the only option for fertility preservation in pre-pubertal males.

## Immature testicular tissue cryopreservation

Since pre-pubertal boys cannot benefit from sperm banking, a potential alternative strategy for preserving their fertility involves storage of testicular tissue in the hope that future technologies will allow its safe utilization. It is important to stress, however, that this strategy is still experimental.

As pre-pubertal testicular tissue contains SSCs from which haploid spermatozoa are ultimately derived, these cells can either be cryopreserved as a cell suspension [19], in the form of tissue fragments [20–22] or even as a whole organ.

It is nevertheless worth noting that, in 20% of Tanner stage II boys, spermiation has already started [23], allowing cryopreservation of haploid gametes that may subsequently be considered for in vitro maturation (IVM), if necessary.

### Cell suspensions

Cell suspensions have been developed with a view to facilitating cryopreservation, as cell heterogeneity in tissue pieces renders tissue freezing more challenging. However, preparation of cell suspensions requires mechanical and/or enzymatic digestion of tissue, compromising cell survival and cell-to-cell interactions necessary for cell proliferation and differentiation [19].

Post-thaw viability of 29–82% has been reported in various animal models [24] and up to 60% in human testicular cell suspensions, regardless of cryoprotective agent [19, 25].

### Tissue pieces

Cryopreservation of testicular tissue pieces may be considered as an alternative method capable of maintaining cell-to-cell contacts between Sertoli and germinal stem cells, and therefore preserving the stem cell niche necessary for their survival and subsequent maturation [26]. Other advantages of this method may be preservation of the Sertoli cells, since there is evidence of their reversion to a dedifferentiated state as a consequence of chemotherapy [9], and Leydig cells, whose preservation may be useful to alleviate the hormonal imbalance caused by cytotoxic therapy [2].

Better survival rates of Leydig cells were obtained when dimethyl sulfoxide (DMSO) was used (80% compared to 50% with 1,2–propanediol [PROH]) [27]. Structural integrity and functional capacity were demonstrated after cryopreservation and culture of pre-pubertal testicular tissue [20, 21].

Because of the complexity of the tissue architecture, cryopreservation protocols must strike a balance between optimal conditions for each cellular type. In addition, problems can arise when extracellular ice forms, as it can cleave tissues into fragments. Furthermore, rapid solute penetration of highly compacted tissue is vital to ensure high final concentrations of cryoprotectant at temperatures that minimize cytotoxicity. Post-thaw survival and seminiferous tubule structure are profoundly affected by both the type of cryoprotectant and freezing rates [28], so optimization of freeze–thawing protocols is mandatory. Dimethyl sulfoxide, rather than ethylene glycol (EG), PROH or glycerol, was shown to better preserve structures within tissue [27, 29] and best maintain tissue capacity to initiate spermatogenesis [30], and slow-programmed freezing to better protect spermatogonial morphology [21].

Two teams have reported freezing protocols for pre-pubertal human testicular tissue, both yielding good structural integrity [20, 21]. Using different cooling and freezing rates, Keros *et al.* observed a difference mainly in terms of survival of spermatogonia, with 94% of intact spermatogonia found after freeze–thawing and culture with their best protocol [21]. This protocol, albeit slightly modified by the addition of sucrose, was further applied to evaluate the functional capacity of cryopreserved human immature testicular tissue (ITT) after orthotopic xenografting [22, 31]. Preservation of spermatogonia (able to proliferate) was demonstrated. An overview of all studies on cryopreservation of ITT is presented in Table 16.2 [20–22, 31].

### Whole testis

Due to the small number of SSCs contained in a testicular biopsy and the small size of a child's testis, it is possible that cryopreservation of a whole testis may be more appropriate, with a view to later organ autografting. Cryopreservation methods for whole testes need to be developed, however, as has been done for whole ovaries [32].

## Fertility restoration after immature testicular tissue cryopreservation

In the light of results obtained from animal studies, frozen diploid precursor cells may provide some hope of fertility restoration in pre-pubertal boys in the absence of haploid gametes. Three approaches may be considered: transplantation of purified cell suspensions back to their own testes; autografting of testicular pieces, testicular cell aggregates or whole testes; or IVM up to a stage at which they are competent for normal fertilization through intracytoplasmic sperm injection (ICSI). The latter option, i.e. IVM, is beyond the scope of this chapter.

None of these approaches have proved efficient or safe in humans as yet. These potential options have mainly been studied in animals, and lessons learned from these studies will be reviewed in detail.

### Testicular germ cell transplantation

In this approach, spermatogenesis is reinitiated after transplantation of isolated testicular stem cells to germ cell-depleted testes. Spermatogonial stem cells (SSCs) are recognized by Sertoli cells and relocate from the lumen onto the basement membrane of seminiferous tubules. Because stem cells have unlimited potential to self-renew and produce differentiating daughter cells, SSC transplantation offers the possibility of long-term restoration of natural fertility.

The technique was first described in 1994 [33]. Testicular germ cells isolated from pre-pubertal mouse testes were injected into the seminiferous tubules of adult mice with Sertoli cell-only syndrome induced by busulfan treatment. Normal donor spermatogenesis, recognized by developing germ cells carrying the lacZ gene encoding β-galactosidase, was initiated and sustained.

Although this approach has yielded healthy progeny displaying the donor haplotype in animals [34], it has not yet proved successful in humans (see Progress towards human clinical application section, below).

## Lessons learned from transplantation of fresh testicular stem cells in animals

### Outcome of the technique

Autologous SSC transplantation has been reported in mice [33], rats [35], pigs [36], goats [37], cattle [38],

**Table 16.2** Overview of studies on cryopreservation of pre-pubertal human testicular tissue

| Reference | Cryoprotectant | (Non) controlled | Freezing rate | Type of evaluation | Outcome (germ cells) | Outcome (endocrine compartment) |
|---|---|---|---|---|---|---|
| Kvist *et al.* [20] | EG 1.5 M Sucrose 0.1 M | Slow controlled | Start: 1°C, −2°C/min to −9°C, hold 5 min + seeding, −0.3°C/min to −40°C, −10°C/min to −140°C, $LN_2$ | Culture 2 weeks | Well preserved STs Presence of intact SG (c-kit$^+$) | Well-preserved interstitial cells Testosterone and inhibin levels similar to fresh tissue |
| Keros *et al.* *[21]* | DMSO 0.7 M | Slow controlled | **Program 1:** Start: 4°C, hold 30 min, −1°C/min to 0°C, hold 5 min, −0.5°C/min to −8°C, seeding, hold 10 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −70°C, $LN_2$ | Culture 24 h | 70 ± 7% ISTs in frozen-cultured tissue (versus 71 ± 7% in fresh tissue and 77 ± 4% in fresh-cultured tissue) 94 ± 1% intact SG in frozen-cultured tissue (versus 93 ± 2% in fresh tissue and 83 ± 1% in fresh-cultured tissue) | Undamaged stromal structure: 80 ± 29% of frozen-cultured samples (versus 99.49 ± 0.88% of fresh samples and 97 ± 2% of fresh cultured samples) |
| | | Rapid controlled | **Program 2:** Start: 4°C, hold 30 min, −1°C/min to −8°C, seeding, hold 10 min, −10°C/min to −80°C, $LN_2$ | | 20 ± 14% ISTs in frozen-cultured tissue 50 ± 43% intact SG in frozen-cultured tissue | Undamaged stromal structure: 29 ± 28% of frozen-cultured samples |
| Wyns *et al.* [22] | DMSO 0.7 M Sucrose 0.1 M | Slow controlled | Start: 0°C, hold 9 min, −0.5°C/min to −8°C, hold 5 min + seeding, hold 15 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −80°C, $LN_2$ | Immediate post-thaw evaluation | 0.71 ± 0.89 SG/ST in frozen–thawed tissue (versus 0.45 ± 0.35 SG/ST in fresh tissue) | Not assessed |
| | | | | Xenografting 3 weeks | 82.19 ± 16.46% ISTs in frozen-grafted tissue (versus 93.38 ± 6% in fresh tissue) 14.5% SG recovery after freezing and grafting | |
| Wyns *et al.* [31] | DMSO 0.7 M Sucrose 0.1 M | Slow controlled | Start: 0°C, hold 9 min, −0.5°C/min to −8°C, hold 5 min + seeding, hold 15 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −80°C, $LN_2$ | Xenografting 6 months | 55 ± 42% ISTs in frozen-grafted tissue 3.7 ± 5.5% SG recovery 21% proliferating SG Differentiation up to pachytene stage of prophase | Signs of steroidogenic activity by 3β-HSD IHC and TEM |

DMSO, dimethyl sulfoxide; EG, ethylene glycol; HSD, hydroxysteroid dehydrogenase; IHC, immunohistochemistry; $LN_2$, liquid nitrogen; SG, spermatogonia; (I)ST, (intact) seminiferous tubule; TEM, transmission electron microscopy.

monkeys [39] and dogs [40]. Restoration of fertility from donor stem cells has only been reported in mice [34], rats [41], goats [37] and chickens [42].

Heterologous transplantation does not appear to be as successful as autologous transplantation, probably because of the phylogenetic distance between species. Rat gonocytes produced mature spermatozoa after xenogeneic transplantation to the testes of mice, but qualitative and quantitative abnormalities of sperm were observed [43]. Abnormal spermatozoa were also found when hamster germ cells were transplanted to mice, probably reflecting the limited ability of mouse Sertoli cells to fully support hamster germ cells [44].

Spermatogonial stem cells from all other mammalian species examined (i.e. rabbits, dogs, pigs, bulls, stallions, non-human primates and humans) were able to colonize the seminiferous tubules of mice and generate colonies of stem cells, but could not differentiate beyond the stage of spermatogonial expansion [45–49]. One study nevertheless demonstrated some early meiotic spermatocytes after transplantation of male porcine germ cells to mice [50]. This suggests that the initial steps of germ cell recognition by Sertoli cells, migration to the basement membrane, initiation of cell proliferation and possibly some early steps of differentiation are conserved among evolutionarily divergent species.

## Efficiency of the technique

The extent of spermatogenesis has been shown to depend on the number of transplanted stem cells, with an almost linear correlation [51], and on the quantity and quality of stem cell niches in the recipient testis [35].

In rodents, the observed colonization rate was no higher than 1/20 SSCs [51], thus showing low colonization efficiency. The colonization rate of slowly cycling type A *dark* spermatogonia in primates was expected to be much lower, estimated to be as low as 0.0015–0.003% in rhesus monkeys [48]. Recipient age appears to have an impact on colonization efficiency, since more and larger spermatogenic colonies were generated in preadolescent recipient mouse testes than in adult testes [52]. Better niche accessibility and niche proliferation due to Sertoli cell multiplication, elements facilitating colony formation and an increase in seminiferous length during testicular enlargement may be involved. This should be taken into account to ensure optimal transplantation time in clinical practice.

## Techniques for SSC enrichment and expansion

Because of the small number of SSCs in a testis (2/10 000 germ cells) [6], the small size of testicular biopsies recovered for fertility preservation and the low efficiency of recolonization after transplantation, increasing the number of SSCs prior to transplantation is essential. Ideally, isolation of pure stem cells would be the most effective method to increase the number of SSCs in a suspension and therefore transplantation efficiency.

Adequate purification will probably be best achieved by cell-sorting techniques, such as magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) based on cell characteristics and membrane antigens. These techniques have already been shown to improve transplantation efficiency in mice [53]. So far, the highest level of SSC enrichment has been achieved based on THY1 expression [54]. As conserved expression of some markers of undifferentiated spermatogonia (PLZF, GFR-α1 and THY-1) exists between mice and non-human primates [48], there is hope that cell enrichment techniques may be extended to humans.

While expansion of pure stem cells in culture appears to be possible, albeit with limited cell proliferation, better results were achieved using culture on feeder layers with a combination of growth factors or applying serial transplantation procedures [55, 56].

So far, strategies for in vitro expansion of SSCs have only proved successful in rodents. Using various growth factors and hormones, a $2 \times 10^{14}$-fold expansion in total neonatal mouse testicular cell number was achieved over ~160 days [56] and, after 2 years, the cultured cells showed $10^{85}$-fold logarithmic proliferation, retaining characteristic morphology and yielding fertile offspring after stem cell transplantation [57].

# Lessons learned from transplantation of frozen testicular stem cells in animals

Since high survival rates do not guarantee preservation of the functionality of frozen–thawed cells, it is important to evaluate their capacity to self-renew and

differentiate through transplantation of cell suspensions. Experiments on human germ cell transplantation were not able to achieve this goal since, after 6 months' xenotransplantation to immunodeficient mice, only proliferative activity was observed [49]. Hence, studies in animals will help us elucidate some important considerations for clinical application.

The potential of frozen murine testicular cells to resume spermatogenesis after transplantation was demonstrated for the first time by Avarbock *et al.* in 1996 [58]. Live birth of offspring achieved after transplantation of frozen testicular cell suspensions provided final proof of successful cryopreservation [59]. Although it appears that the functional capacity of mouse SSCs may be compromised by cryopreservation [60], this was not observed by Kanatsu-Shinohara *et al.* [59]. Moreover, rhesus SSCs retained normal colonization capacity after freezing and transplantation to mice [48], suggesting that possible functional impairment due to cryopreservation involves germ cell differentiation rather than their ability to recolonize stem cell niches.

## Progress towards human clinical application

In humans, preclinical in vitro studies using cadaver or surgically removed testes have demonstrated the feasibility of transplanting germ cell suspensions into testes. Fifty to seventy percent of seminiferous tubules were filled by means of intratubular injection [19] or injection into the rete testis, with needle placement controlled by ultrasonography [61].

A clinical trial was initiated in Manchester (UK) in 1999 to evaluate germ cell transplantation in cancer patients but, as far as we know, no information is available on the fertility of these patients [62]. Drawing conclusions from this trial will nevertheless be problematic, as endogenous spermatogenesis and spermatogenesis issuing from transplanted cells will not be distinguishable.

### Testicular tissue grafting

Testicular tissue grafting involves transplantation of SSCs with their intact niches and thus within their original microenvironment. Since testicular tissue grafting has not yet been reported in humans, available data will be reviewed on the basis of observations made in animals.

To date, haploid germ cells isolated from mouse testis homografts and rabbit testis xenografts have been used with ICSI to generate offspring [63]. Xenogeneic rhesus sperm generated in host mice have also been shown to be fertilization competent, allowing in vitro embryo development at a rate similar to that reported for *in situ* rhesus testicular sperm [64]. In view of these encouraging results in animals, there is every hope that it will be possible, in the near future, to autograft cryopreserved testicular tissue of patients rendered sterile after fertility-threatening therapies and restore their fertility.

## Lessons learned from transplantation of fresh testicular tissue in animals

Grafting of testicular tissue from several mammalian species into immunodeficient mouse hosts has resulted in varying degrees of donor-derived spermatogenesis. Complete spermatogenesis following testicular grafting has been reported in mice, rabbits, hamsters, pigs, goats, cats, bovines, horses and sheep [63–70], as well as macaques [64, 71]. By contrast, germ cell differentiation blockage was observed in marmosets [30, 65, 72].

The mechanisms underlying these species-specific differences in spermatogenic differentiation remain unknown, but some hypotheses can be proposed.

First, differences between host and donor gonadotropic hormones [73] may lead to inefficient interaction between murine gonadotropins and grafted donor testicular tissue. Supplementation with exogenous gonadotropins could therefore be useful. Indeed, xenografts of ITT from rhesus monkeys to mice treated with exogenous gonadotropins showed some degree of sperm differentiation, compared to blockage at the spermatogonial level observed in untreated mice [71]. However, observations from two different studies do not support this hypothesis, since autologous grafts of marmoset tissue [72] and xenografts of marmoset and horse ITT to gonadotropin-supplemented recipient mice [69] showed blockage in germ cell differentiation. These conflicting results suggest that species-specific differences in gonadotropins are not the only explanation for differentiation impairment.

Second, as suggested by studies on testicular tissue xenografts from macaques and marmosets, species-specific structural differences in seminiferous tubule organization [74], resulting in modified paracrine

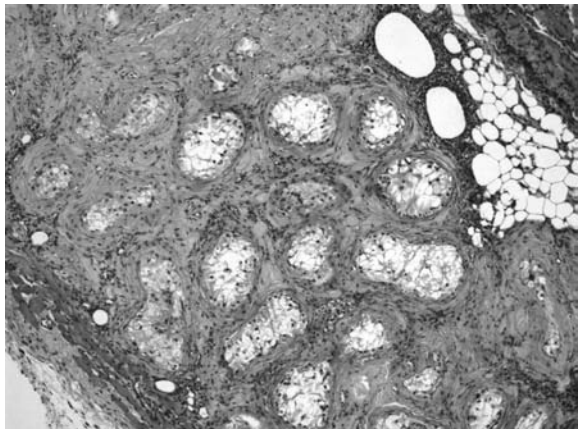

**Figure 16.1** Histological appearance (hematoxylin/eosin sections) of donor testicular tissue from a 44-year-old man after 3 weeks' orthotopic xenografting at ×200 magnification. Most tubules show degenerative changes, i.e. sclerosis, while the rest contain mainly Sertoli cells. See plate section for color version.

interactions, might explain differences in germ cell differentiation within grafts.

Third, the initiation and extent of differentiation may be influenced by the stage of germ cell development and intensity of spermatogenesis at the time of grafting. Indeed, complete spermatogenesis was not reported in xenografted tissue when donor testicular tissue contained post-meiotic germ cells at the time of grafting in any species, including humans, and most grafts regressed or contained degenerated tubules (Figure 16.1) [65, 69, 75, 76].

The reasons for the poor outcome of adult testicular tissue xenografts are so far unknown. However, studies in rodents have suggested that adult tissue could be more sensitive to ischemia than immature tissue, and that hypoxia related to the grafting procedure may be involved [65]. This hypothesis was supported by studies in bovines, showing higher expression of some angiogenic factors in grafts from younger donors [77]. Furthermore, pre-treatment of testicular tissue with vascular endothelial growth factor (VEGF), a potent angiogenic factor, was found to increase the number of tubules containing elongating spermatids [78].

Nevertheless, the angiogenesis hypothesis is probably insufficient to explain this poor outcome, since donor age-dependent variations in germ cell differentiation have also been observed in immature donors [68, 71]. Variations in Sertoli cell maturation at the time of grafting, or their developmental susceptibility to the detrimental influence of endocrine disruption due to the xenografting environment, may account for the inability of these cells to support germ cell differentiation, and may thus be involved in the age-dependent variations found [68, 71]. Donor age-dependent differential gene and subsequent protein expression in donor tissue prior to grafting may also be implicated [77].

Besides causing spermatogenic differentiation impairment, xenografting has been shown to be inefficient in some species. Indeed, only 5–10% of seminiferous tubules in xenografts produced elongated or elongating spermatids in bulls [68], kittens [67] and horses [69]. Furthermore, in non-human primate testicular tissue grafts, only 2.8–4.0% of tubules contained mature sperm [64, 71]. The reasons for this low spermatogenic efficiency need to be understood in order to improve the success of this approach.

Initial germ cell loss, as reported in bovine and monkey xenografts [71, 79], could explain these poor results. Decreased expression of glial cell line-derived neurotrophic factor (GDNF), involved in germ cell self-renewal, has been described in grafts [77], suggesting that the grafting procedure itself could negatively influence the number of germ cells. However, tissue culture performed prior to xenografting to increase the number of SSCs did not result in a higher percentage of seminiferous tubules with elongating spermatids at the time of graft removal [78], indicating that other factors may be responsible for the low spermatogenic efficiency.

## Lessons learned from transplantation of frozen testicular tissue in animals

An overview of studies on cryopreserved testicular tissue grafting in various animal models was recently reported by Geens *et al.* [24]. In rodents, cryopreservation of ITT led to the birth of healthy offspring [63]. There is therefore every hope that this approach can be extended to humans.

A number of studies in animals designed to evaluate the effect of freezing on the functional capacity of germ cells have shown that freezing does not appear to affect the functional capacity of frozen germ cells on a qualitative basis [29, 30, 63, 65, 66, 80]. Loss of SSCs after cryopreservation was nevertheless suggested, since Ohta and Wakayama reported lower colonization efficiency after grafting frozen–thawed testicular pieces [80].

## Lessons learned from xenotransplantation of fresh human testicular tissue

Very few studies have been published on xenotransplantation of human testicular tissue [75, 81]. Adult testicular tissue grafting has yielded poor results, showing mainly sclerotic seminiferous tubules [75, 81] and some isolated spermatogonia in 21.6–23.1% of grafts [75].

Grafting of human ITT from pre-pubertal boys [82] did not result in complete spermatogenesis, although graft and germ cell survival were shown to be more favorable than in mature tissue grafts. Goossens *et al*. observed mainly Sertoli cell-only tubules and just a few surviving spermatogonia 4 and 9 months after grafting, constituting considerable spermatogonial loss [82].

## Lessons learned from xenotransplantation of frozen human testicular tissue

No studies have reported xenografting of cryopreserved adult testicular tissue in humans and only two have been published on cryopreserved ITT xenotransplantation in humans [22, 31]. Grafts were performed orthotopically to immunodeficient mice. After grafting frozen–thawed cryptorchid tissue for 3 weeks, 14.5% of the initial spermatogoniaI population survived, with 32% of these cells showing proliferative activity, not significantly different from the 17.8% in fresh tissue. The number of Sertoli cells was unchanged and 5.1% were proliferative compared to 0% in fresh tissue. Raised follicle stimulating hormone (FSH) levels in the castrated mice, the removal of some inhibitory mechanisms that normally operate in quiescent immature testes and/or other paracrine factors were suggested to play a role in the Sertoli cell multiplication. In order to study the capacity of frozen SSCs to self-renew and differentiate, long-term grafts of normal immature tissue were performed. After freeze–thawing and 6 months' xenografting, 3.7% of the initial spermatogonial population remained, with 21% of these cells showing proliferative activity.

Since considerable loss of spermatogonial cells occurred, it is essential to evaluate to what extent cryopreservation itself is implicated. Freezing does not appear to have a major impact. Indeed, no decrease in spermatogonial cell numbers between fresh and frozen–thawed testicular pieces [22] and a very high survival rate ($94 \pm 1\%$) of spermatogonia after freezing and culture [21] were observed. Furthermore, regarding the effect of cryopreservation on the differentiation capacity of human SSCs, the remaining spermatogonia retained the ability to reinitiate spermatogenesis, although normal differentiation beyond the prophase of the first meiosis could not be proved [31]. Indeed, spermatid-like structures were detected (Figure 16.2a–c), albeit slightly smaller than control spermatids ($P = 0.045$), but these structures did not show characteristic markers of postmeiotic cells or acrosome development by immunohistochemistry (IHC).

As shown in Figure 16.3, signs of preservation of the steroidogenic capacity of Leydig cells were also observed [31].

## Testicular cell grafting

Isolated cell grafting was first described in cotransplantation experiments where Sertoli cells were used for their immunoprotective properties. The capacity of dissociated Sertoli and myoid cells to reaggregate and form seminiferous cords after xenotransplantation was first demonstrated by Dufour *et al.* [83]. This approach is challenging, however, since the different cell types, including Leydig cells, have to form functional three-dimensional cell associations after transplantation in order to produce a supportive microenvironment for spermatogenesis, whereas in tissue grafts these are already formed.

Isolated cells from immature mammelian testes were nevertheless able to reproduce partial or complete spermatogenesis and initiate steroidogenesis after ectopic or orthotopic grafting.

Moreover, spermatogenesis in grafts originating from single-cell pellets was shown to be morphologically identical to spermatogenesis occurring in grafts of intact testicular tissue in pigs [84]. Offspring were finally obtained after mouse germ cell transplantation in ectopically reconstituted tubules created from isolated murine testicular cells [85].

Studies assessing germ cell differentiation and/or Leydig cell functionality after xenografting of isolated immature testicular cells in mouse recipients are summarized in Table 16.3 [86–88].

In the light of these animal studies, this approach looks promising from a clinical perspective. Indeed, besides allowing reconstitution of a functional stem

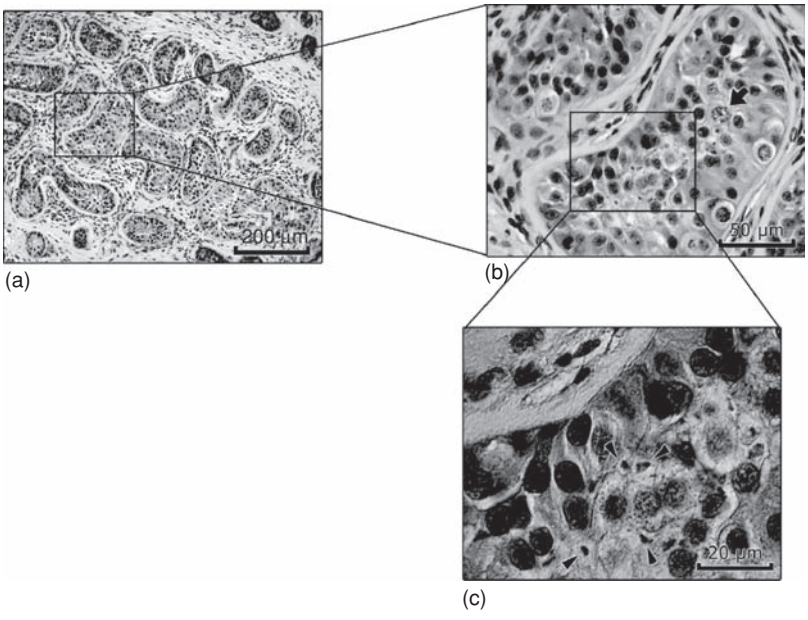


**Figure 16.2** Histological appearance (hematoxylin/eosin sections) of donor testicular tissue from a 12-year-old boy (a) after 6 months' orthotopic xenografting at ×200 magnification; (b) showing pachytene spermatocytes (arrow) and spermatid-like cells (inset) at ×400 magnification; and (c) spermatid-like cells at ×1000 magnification. See plate section for color version.

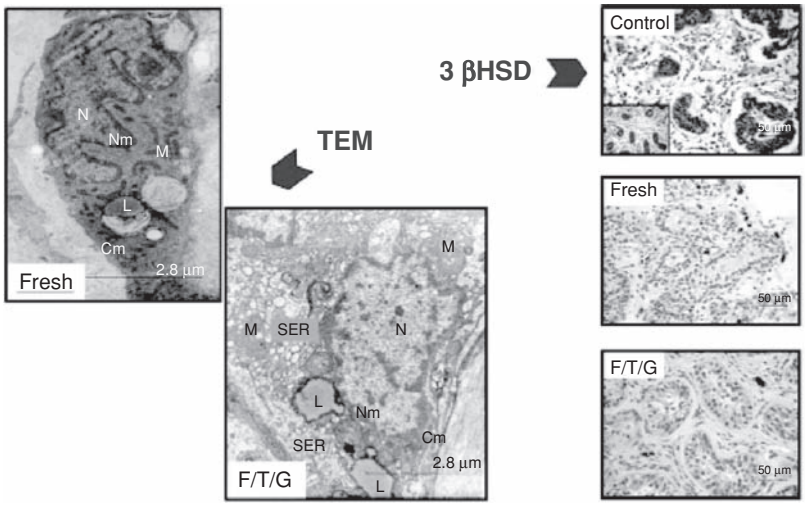


**Figure 16.3** Steroidogenic activity in Leydig cells evaluated by transmission electron microscopy (TEM) (left) and immunohistochemistry (IHC) (right). The TEM shows fresh and frozen/thawed/grafted Leydig cells showing intact structures of nuclear and cytoplasmic components and activity. Magnification ×12 000. Bm, basement membrane; Cm, cell membrane; F/T/G, frozen, thawed and grafted for 6 months; L, lipid droplets; M, mitochondria; N, nucleus; Nm, nuclear membrane; SER, smooth endoplasmic reticulum: site of conversion of pregnenolone to testosterone. The IHC shows fresh and frozen/thawed/grafted Leydig cells that are stained for 3 β-hydroxysteroid dehydrogenase (3 βHSD), converting pregnenolone to progesterone. See plate section for color version.

cell niche, this technique offers the possibility of using cell sorting methods before grafting and could therefore be beneficial when testicular tissue is potentially contaminated with cancer cells.

In addition, it may help to elucidate factors regulating spermatogenic events after grafting and potential reasons for loss of spermatogenic activity after a gonadotoxic insult [87].

## Whole testis transplantation

The first convincing demonstration of human testis transplantation was reported in 1978 by Silber [89]. An anorchid man was grafted with a testis from his genetically identical twin brother and, after vessel microanastomosis and vasovasostomy, serum testosterone levels increased and 15 million spermatozoa per milliliter of ejaculate were observed.

**Table 16.3** Studies on immature testicular cell xenografting

| Reference | Donor species | Recipient species | Graft localization | Tubule reconstitution | Sperm differentiation | Steroidogenesis |
|---|---|---|---|---|---|---|
| Gassei *et al.* [86] | Rat (after culture) | Nude mouse | Back skin | Yes | Few putative spermatogonia<br>No further differentiation | IHC identification of Leydig cells<br>Production of bioactive testosterone[a] |
| Kita *et al.* [85] | Mouse, rat | Nude/SCID mouse | Back skin | Yes | Round spermatids<br>Offspring from mouse-testis-cell transplants | Not assessed |
| | Pig | | | Yes | Not assessed | |
| Honaramooz *et al.* [84] | Pig | Nude/SCID mouse | Back skin | Yes | Complete spermatogenesis | IHC identification of Leydig cells<br>Production of bioactive testosterone[a] |
| Arregui *et al.* [70] | Sheep | Nude mouse | Back skin | Yes | Complete spermatogenesis | Production of bioactive testosterone[a] |
| Zhang *et al.* [87] | Bovine (after culture 3–7 days) | Nude mouse | Back skin<br>Testis | Yes<br>Yes | No germ cells<br>No germ cells | Not assessed |
| Watanabe *et al.* [88] | Neonatal pig | Nude/SCID/NC mouse | Back skin | Yes | Complete spermatogenesis | Not assessed |

[a] Production of bioactive testosterone assessed by increase in seminal vesicle weight.
IHC, immunohistochemistry.

More recently, transplantation of rat testes showed active spermatogenesis in 42% of fresh isotransplants but, after transplantation of intact cryopreserved testes, none was found to be functional [90].

# Safety issues

## Cancer cell contamination

The most important, life-threatening concern of spermatogonial transplantation is the risk of reintroducing malignant cells. Indeed, the majority of pediatric malignancies metastasize through the blood, thus carrying a high risk of malignant contamination of the testes. The risk is greater with hematological cancers, as the testes can act as sanctuary sites for leukemic cells. Indeed, it has already been shown that as few as 20 leukemic cells injected into a testis can induce disease relapse [91].

Germ cell isolation and cell-sorting techniques enabling complete purification of SSCs therefore need to be validated before safe transplantation can be contemplated. While cell-sorting methods have shown promising results in animal studies, the same cannot be said of humans [92].

One of the reasons for suboptimal cell sorting may be that the surface antigens are shared by other stem cells, namely hematopoietic stem cells involved in hematological cancers. Immunophenotyping malignant cells from each patient, followed by inclusion of patient-specific cancer antigens for cell sorting, should therefore improve the success of the technique. For this purpose, we strongly advise storing patient blood and/or tumor samples before therapy.

As no specific marker exclusive to SSCs has yet been identified, allowing positive selection of these cells through cell sorting techniques, further research on surface markers should focus on the complete elimination of cancer cells from cell suspensions before sorted preparations can be safely transplanted.

Cancer cell contamination is also a major concern in tissue autografting. Since it has been

reported that leukemic cells can survive cryopreservation/xenotransplantation and increase the incidence of generalized leukemia in the nude mouse host [93], testicular tissue autografting after cure can only be considered for patients in whom there is no risk of testicular metastases or who have undergone gonadotoxic therapies for non-malignant disease.

## Infectious transmission

Due to the risk of infectious transmission from animals to humans, testicular xenografting should not be considered for reproductive purposes at present. This approach is nevertheless useful for the evaluation of the functional capacity of germ cells and should therefore form part of the assessment of germ-cell cryopreservation protocols, for the understanding of testicular physiology and pathophysiology and for testing malignant contamination of tissue before autografting.

## Birth defect risks

Goossens *et al*. recently reported smaller litter size, significantly lower fetal weight and reduced length in first generation mouse offspring after germ cell transplantation, suggesting imprinting disorders [94]. Further investigation is therefore required to elucidate the underlying reasons before autotransplantation can be safely introduced into clinical practice.

## Ethical concerns

Learning that a child has cancer is devastating for all concerned and treatment needs to begin quickly, leaving very little time for the impact of possible future sterility to sink in. However, the inability to father one's own genetic children might have a huge impact on the psychological well-being of patients in adulthood [10], so it is crucial to inform them of the potential consequences of their therapy on future fertility. Ethical concerns have been expressed about ITT cryopreservation, highlighting the importance of the risk/benefit balance [95]. Because of the small size of testes from pre-pubertal children, immature gonadal tissue sampling may be considered too invasive a procedure, which must therefore be done for good reason. However, in the two available studies on testicular tissue harvesting in young cancer patients [21, 22], no major surgical complications occurred during testicular biopsy. Mean biopsy volume was about 5% of testicular volume which, according to morphological studies [6], should provide enough germ cells for fertility preservation. Furthermore, in a follow-up study of cryptorchid boys who had undergone testicular biopsy during orchidopexy, no adverse long-term effects were reported [96]. Regarding general anesthesia, since this biopsy is generally performed under the same anesthesia as that used for placement of the central line for chemotherapy, there is no additional risk involved.

When considering the benefits of tissue harvesting, the safety and effectiveness of fertility preservation and restoration procedures are essential issues. Children and their parents should be informed of the experimental nature of this approach and the fact that there is no guarantee of fertility restoration. Parental consent and the child's ascent, meaning he was given the opportunity to discuss the procedure, should be sought. As obtaining fully informed consent from children is difficult, substituted consent from parents should for now be limited to the safekeeping of tissue [95, 97].

# Conclusion

Providing young people undergoing gonadotoxic treatment with adequate fertility preservation strategies is a challenging area of reproductive medicine, but every patient should be given the chance to consider fertility-sparing options because the detrimental effect of such therapy on gonadal function remains unpredictable. Hormonal or cytoprotective drug manipulation aimed at enhancing spontaneous recovery of spermatogenesis remains a possibility for the future. Preservation of SSCs offers the prospect of several realistic applications, although none is feasible in humans at this point in time. Future advances in fertility preservation technology rely on improved understanding of the cryobiology of gonadal tissue and cells.

Before considering fertility restoration options, patient selection is essential, since risks vary according to disease. No single (or simple) algorithm can so far summarize all the possible strategies for fertility preservation and restoration in case of gonadotoxic therapy in pre-pubertal boys, but the most appropriate course of action may be selected according to the scheme shown in Figure 16.4. Over the next few years, research should focus on how to extend successful experiments in animals to young boys and on the identification of the ideal microenvironment for SSC development. Resolving numerous important technical issues discussed in this chapter should lead to safe and efficient methodologies for fertility restoration

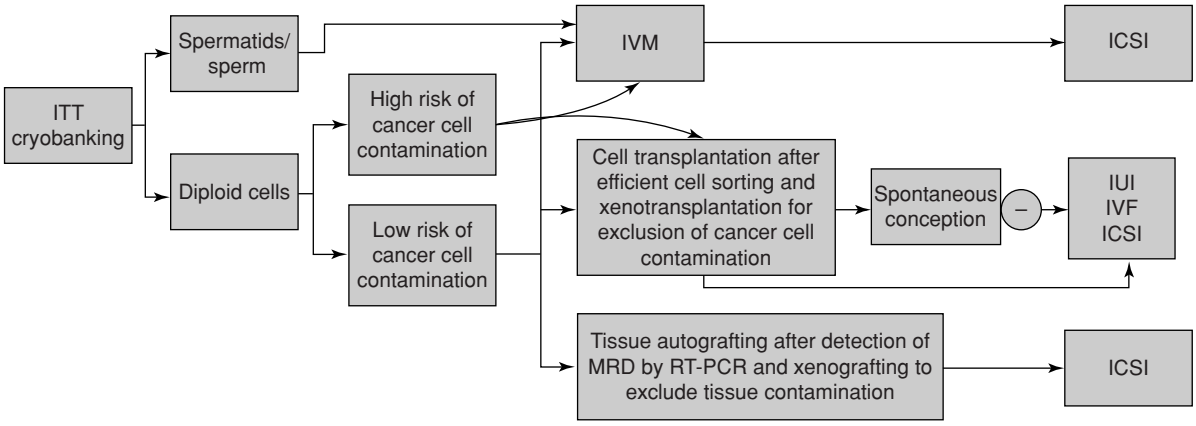


**Figure 16.4** Fertility restoration strategy after gonadotoxic therapy in pre-pubertal boys. ICSI, intracytoplasmic sperm injection; ITT, immature testicular tissue; IVF, in vitro fertilization; IUI, intrauterine insemination; IVM, in vitro maturation; MRD, minimal residual disease; RT-PCR, real-time polymerase chain reaction.

after storage of preserved gametes, and the development of ethically accepted pilot protocols, which will then need to be submitted for further ethical approval before definitive and universal clinical implementation. Until then, samples should at least be banked after providing careful counseling and obtaining informed consent, making sure the patient understands there is no guarantee of success. Preservation of testicular tissue from today's pre-pubertal patients will allow them to consider various fertility restoration options that will emerge in the next 20–30 years, giving them hope of fathering children with their own genetic heritage.

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Chapter 17

# Assisted reproductive techniques and donor sperm in cancer patients

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## Introduction

Recent advances in the treatment of cancer have lead to greater longevity and an increased recognition that quality of life including paternity is significant issues for cancer survivors. We will focus primarily on patients with testicular cancer and lymphoma that generally affects younger patients in the reproductive window with an excellent overall survival. However, one must realize in our modern society the age of desiring paternity has increased due to postponement of marriage as well as for other social reasons. Therefore, this chapter will focus not only on those who completed chemotherapy as children but adult cancer patients as well.

## Chemotherapeutic effects

The effect of chemotherapeutics on spermatogenesis varies by both the drug administered as well as the cumulative dosage and will be discussed in other chapters. However, it is obvious that due to their high proliferative index, chemotherapeutics will be toxic to germ cells while Leydig cells seem more resistant to the effect of systemic chemotherapy. Therefore, serum levels of testosterone may be within normal limits and yet impairment in sperm production may lead to infertility.

There is no diagnostic test to tell whether spermatogenesis will return after chemotherapy. Men with return of sperm to the ejaculate may even conceive naturally and all reproductive options are open to them. However, a focus on men who are rendered azoospermic after therapy remains a clinically important subgroup of patients who are candidates for treatment. Up to 13.8% of men are azoospermic prior to chemotherapy [1, 2]. What are we to do with this population? This chapter will discuss the treatment options available to azoospermic males prior to chemotherapy as well as post-chemotherapy azoospermia. Though multiple advanced reproductive techniques are now available to the post-chemotherapy population, it remains an obvious recommendation that men bank sperm prior to any chemotherapy or gonadal radiation treatment, optimizing options for effective treatment.

## Sperm banking before chemotherapy/radiotherapy

In most cases, sperm collection and cryopreservation of sperm is a simple task prior to the initiation of chemotherapy or radiation therapy. In men with normal ejaculation, sperm cryopreservation involved the collection of a masturbatory sample that is subsequently frozen. Barriers to universal sperm banking are variable but may include inadequate counseling by physicians, inability to afford sperm banking, limited sperm quality for cryopreservation, pre-pubertal or young emotional age of the patient, religious restrictions on sexual activity or the sense of urgency to begin cancer treatment.

Unfortunately, low rates of sperm banking prevail. One study that examined young adult survivors of childhood cancer found that nearly 60% reported uncertainty about their fertility status, and only half recalled a healthcare provider discussing potential reproductive problems associated with treatment [3]. In 2002, a survey of cancer patients treated at two major cancer centers revealed that only 51% of respondents had been offered sperm banking and only 24% of respondents actually banked sperm [4]. A lack of

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information was the most common reason for not banking sperm in up to 25% of patients in this study [4]. Surveys in the USA [5, 6], UK [7], Australia and New Zealand [8] show that many oncologists do not routinely provide information on and referrals for banking sperm to teenagers and young men, despite claiming that sperm banking should be offered to all men at risk of infertility from cancer treatment. Sperm banking seemed to be offered less when the patients had aggressive disease or poor prognosis [6]. But even with successful banking, rates of subsequent utilization of cryopreserved sperm appear to be low. Several recent reports from sperm banking facilities concur that <20% of men who store sperm before cancer treatment end up using it to try to conceive [2, 9–11]. Of course, such numbers could be artificially suppressed by: (1) lack of survivorship for some patients who elect to bank sperm; (2) the long delay between medical therapy for cancer and subsequent election to have children; (3) recoverability of sperm production for some men despite toxic treatment regimens; and (4) the lack of insurance support for assisted reproductive treatments in many areas of the USA.

Therefore, all men should be offered and the majority should undergo sperm banking. For the minority of patients who are azoospermic at the time of presentation, more advanced sperm retrieval techniques may be needed to cryopreserve sperm.

## Chance of recovery of spermatogenesis

The chance of recovery of spermatogenesis depends on the chemotherapeutic regimen as well as the baseline function of the patient. Notably, alkylating agents such as cyclophosphamide have been the most extensively studied in this group of drugs and seem to have the most dramatic reproductive effects. However, sperm will return to the ejaculate in numbers sufficient for natural conception in many if not most patients depending on the chemotherapeutic regimen, radiation treatment, surgical treatment and baseline semen parameters. For patients with return of sperm to the ejaculate, reproduction may involve spontaneous conception to intrauterine insemination (IUI) to in vitro fertilization (IVF). However, Schmidt *et al.*, in a review of 67 cancer survivors, found that 57% of men were azoospermic after chemotherapy [12]. The rest of this chapter will focus on the treatment options available to those who remain persistently azoospermic or severely oligospermic.

At present, only crude measures of predicting the return of spermatogenesis after chemotherapy are available. During chemotherapy, follicle stimulating hormone (FSH) levels invariably increase. Kader and Rostom [13] found that persistently elevated levels of FSH at 2 years after chemotherapy is associated with a higher chance of azoospermia. It is worth noting, however, that in our micro-dissection testicular sperm extraction (mTESE) series we have not found FSH to be a predictor of sperm retrieval (to be discussed later) [14]. Otherwise, we eagerly await better markers that may indicate return of spermatogenesis.

## Treatment options for azoospermic men after chemotherapy

Traditionally the patient who presents with azoospermia after chemotherapy has been considered sterile if they did not bank sperm prior to chemotherapy. However, advances in advanced assisted reproductive techniques have enabled even these men to successfully father children with newer techniques for testicular sperm extraction. The realization that the testicle is not uniform in terms of spermatogenesis, has allowed retrieval of sperm from those men once deemed sterile. With the introduction of intracytoplasmic sperm injection (ICSI), we now have the ability to enable conception with very low numbers of sperm. For the man who is azoospermic after chemotherapy there remains a number of choices for reproduction, including the use of previous cryopreserved sperm as well as use of fresh sperm retrieved either by electroejaculation or testicular sperm extraction. Finally, if a patient is unable or unwilling to successfully pursue any of the previous options the option of donor sperm remains.

### Use of cryopreserved sperm

The existence of a previously cryopreserved sperm greatly simplifies the algorithm for the post-chemotherapeutic azoospermic man and, essentially, the couple can go directly to IVF. The outcomes are good for cryopreserved sperm. Hourvitz reported on 118 couples undergoing 169 IVF–ICSI cycles at the Weill Cornell [15]. From 1994 to 2005, using cryopreserved sperm and ICSI, there was a fertilization rate (per injected egg) of 77.6%, a clinical pregnancy rate of 57% (96/169) and a 50% delivery rate (85/169). As a historical control, a similar population was evaluated from 1992 to 1994 at the

same institution prior to the routine use of ICSI. Using conventional IVF, the fertilization rate was 32% and the delivery rate was 24% (13/54) [15]. Agarwal *et al*., in a retrospective study of 29 patients, reported the outcome of use of cryopreserved sperm from men with various malignancies [11]. They showed that with assisted reproductive technology (ART), couples with cryopreserved sperm prior to cancer therapy can be successfully treated to achieve pregnancy. Schmidt *et al*., in a study of patients presenting to a Danish fertility clinic, noted that 22/35 live births in their series were due to the use of cryopreserved sperm [12]. Thus, the use of cryopreserved sperm is both viable and successful.

## In the patient who is azoospermic prior to chemotherapy

Patients with leukemia, lymphoma and testicular cancer often present with suboptimal semen parameters [16–19]. Whether one type of malignancy presents with poorer outcomes is controversial [1, 20, 21]. However, it is notable that up to 13.8% of cancer patients presenting to sperm banks are azoospermic [1, 2]. For the patient azoospermic prior to chemotherapy, there is an even greater urgency with regard to the expediency of treatment. Time is critical and these patients must balance the urgency of getting necessary chemotherapy with the additional worry of successful preservation of spermatozoa. Any fertility treatment must be of minimal morbidity limiting any delay of the patient from the needed chemotherapeutics. For these patients, there is the option of cryopreservation of testicular spermatozoa from normal testis, cryopreservation of spermatozoa from the diseased testis, or preserving epididymal or vasal sperm (for obstructed patients). It is likely that in this setting, yield of spermatozoa from any of these sperm retrieval techniques may be low. The advent of ICSI has made treatment of these patients feasible, since only small numbers of sperm are needed for successful IVF.

Sperm extraction is possible from the contralateral normal testis or from the affected testis (if retrieval is done after separation of the testis from the patient) for testicular cancer at the time of orchiectomy. In patients with lymphoma, sperm extraction can be done in either one side or both testes simultaneously prior to chemotherapy. In 2003, Schrader *et al*. reported 31 men with either testicular cancer or lymphoma [22]. In testicular cancer patients, the TESE sample came from the contralateral testicle, which was also evaluated for concomitant carcinoma *in situ* (CIS). The lymphoma patients underwent bilateral TESE. The sperm retrieval rate was 43% (6/14) in testicular cancer and 47% (8/17) in patients with Hodgkin's or non-Hodgkin's lymphoma [22].

There also is the possibility of vasal or epididymal sperm extraction after orchiectomy from the orchiectomy specimen. Baniel and Sella reported three azoospermic patients with testicular cancer who at the time of radical orchiectomy had vasal and epididymal sperm preserved [23]. After the specimen had been resected, sterile extraction of sperm with cryopreservation of sperm from the vas and epididymis was done. They reported two pregnancies from three couples [23].

Preserving tissue or sperm from the tumor containing testis can be done on a separate sterile field ("a backtable") after orchiectomy or, in cases of partial orchiectomy, the surrounding normal testicular parenchyma can be examined and sent for cryopreservation. A number of groups have reported on these techniques in a limited number of patients with good success at sperm retrieval [24–26].

## Use of spermatozoa from men who have received chemotherapy or radiation

Men who have received chemotherapy, and even men who had non-gonadal radiation therapy, will have increased rates of sperm aneuploidy for 6 months or more after treatment. A variable increase in sperm DNA fragmentation has also been observed after chemotherapy, much of which will return to baseline within 1 year after treatment. Some studies have suggested an increase in birth defects for offspring of men treated with chemotherapy or non-gonadal radiation within the past year. Taken together, these data suggest caution in suggesting that patients attempt to have children early after chemotherapy or radiation treatment.

## In the patient who is anejaculatory after chemotherapy and is oligospermic or normospermic

Any retroperitoneal surgery such as retroperitoneal lymphadenectomy may affect the sympathetic chain or structurally compromise the bladder neck and may

affect antegrade ejaculation. Partial ejaculatory function may be preserved depending on the degree of nerve sparing. The preservation of ejaculatory function obviously has a tremendous impact on fertility rates. In Norwegian testicular cancer survivors who had chemotherapy, those with intact antegrade ejaculation have an 83% paternity rate, while rates for paternity were only 10% in the anejaculatory group [27].

An option for anejaculatory patients is to undergo electroejaculation. This is a procedure generally performed under general anesthesia in the sensate patient. We do not routinely catheterize the patient prior to electroejaculation. The patient is placed in the lateral decubitus position. Anoscopy is performed to confirm that the rectum is empty and no rectal mucosal abnormalities are present. The rectal probe is inserted completely into the rectum with the electrodes oriented anteriorly, over the prostate and seminal vesicles. Stimulation is carried out with a standard electrical stimulation system. The pattern of electrical stimulation has been empirically evaluated but appears to work best with a gradually increasing voltage "peaked" sine wave stimulation that is abruptly ceased, with at least 5–7 s delays between stimulations. The procedure is also monitored by observation of penile tumescence and rectal temperature. Typically, penile tumescence is noted first, followed by seminal emission. When seminal emission ceases, rectal temperature of 38°C is observed, or a maximum of 30 volts is attained, then electrostimulation is stopped. Anoscopy is performed again to insure that there is no rectal mucosal injury, which is a potential complication of this procedure. The patient is turned supine and urethral catheterization is carried out. An initial retrograde specimen is diluted in human tubal fluid (HTF) buffered with HEPES and plasmanate, pH 7.4, and sent for immediate processing, as is the antegrade ejaculate. The bladder is then irrigated with HTF, and this second retrograde specimen is sent for immediate processing as well.

Ohl *et al.* performed electroejaculation in 24 testicular cancer patients (23 of which had undergone retroperitoneal lymphadenectomy) and observed seminal emission in all 24 patients [28]. Greater than 10 million motile and progressive sperm were obtained in 88% (21/24) of patients. In total, 17 couples underwent IUI and the overall cycle fecundity rate was 9%. Seven clinical pregnancies were detected and there were five live births. Electroejaculation has also been successfully combined with IVF (and obtained a 53% fertilization rate [29]) as well as IVF/ICSI with a 75.5% fertilization rate[30]. At Weill Cornell, we have reported a fertilization rate of 75.5%, a clinical pregnancy rate of 56% per retrieval and an implantation rate of 33% per embryo [30].

In 1998, Rosenlund *et al.* looked at 17 couples treated for testicular cancer where most (14/17) received chemotherapy and most patients acquired sperm through electroejaculation [31]. They employed IVF or ICSI and had a fertilization rate of 55–57% in both groups, and the ongoing pregnancy rate for the whole cohort was 57% per cycle [31]. The study demonstrated that treated testicular cancer patients can successfully undergo ART with electroejaculated spermatozoa.

## In the patient who is azoospermic after chemotherapy

Azoospermia after chemotherapy can be due to the patient's chemotherapeutic regimen, the use of radiation, the extent of surgery, the disease itself, the baseline function of the patient or any combination of the aforementioned factors. While these men were once considered sterile, the use of advanced reproductive techniques has enabled paternity in a subset of this population. Specifically, the realization that the testis is not uniform and that there may be small pockets of spermatogenesis in these patients has enabled us to retrieve sperm in patients with non-obstructive azoospermia using mTESE [32].

For men who are azoospermic after chemotherapy, spontaneous recovery may occur in at least a subset of patients within 2–8 years. For men treated with alkylating agents, the duration of azoospermia may be longer, so a period of observation prior to attempted testicular sperm extraction is recommended. For men treated with platinum-based regimens, most men who will have sperm return to the ejaculate can have sperm detected within 2 years. As in any patients with non-obstructive azoospermia, percutaneous aspirations or biopsies, while possible, are more likely to yield low numbers of sperm and require multiple treatments. In our view, the low yield, uncertainty of sperm retrieval and intratesticular bleeding/scarring make these procedures less favorable, especially in this patient population with multiple insults to spermatogenesis. The risk of testicular injury along with low spermatozoa yields led to the development of mTESE [32]. Our data

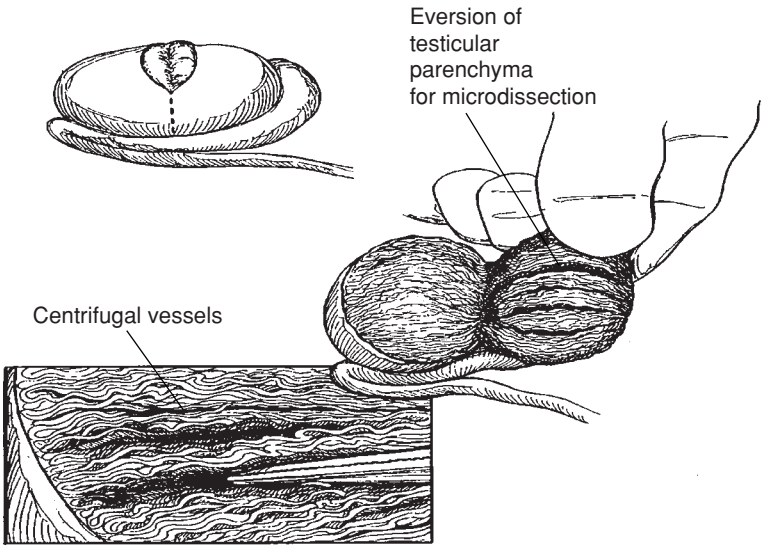


**Figure 17.1** A transverse incision is made in the testis and the testicular tissue is everted and micro-dissected for thorough inspection of all tubules.

and that of others suggest that mTESE yields the highest sperm retrieval rate in this population.

Evaluation prior to mTESE includes a through history, sexual history, chemotherapy history physical exam and hormonal profile. On physical exam, attention is paid to the fullness of the epididymis as well as testicular volumes. The technique of mTESE is as follows. This technique involves placing a wide incision in the tunica albuginea in an avascular region and eversion of the testicular parenchyma for microdissection (Figure 17.1). With high power magnification, subtunical vessels as well as intratesticular vessels can be identified and preserved. Microscopic dissection and direct examination of seminiferous tubules allow identification of the rare regions that contain sperm in men with non-obstructive azoospermia (NOA). The tubules with spermatozoa are wider and more opaque than the fibrotic Sertoli cell-only tubules (Figure 17.2). Overall, mTESE has been shown to result in a higher number of sperm harvested, increased chance of retrieving sperm and decreased testicular tissue removed [32, 33]. The only predictor of successful treatment is the most advanced stage seen on biopsy and not the predominant stage [34]. Testicular volume, serum FSH levels and the etiology of NOA appear to have little or no effect on the chance of sperm retrieval [14, 34, 35]. Postoperative ultrasound has demonstrated fewer acute and chronic changes after micro-dissection as compared to conventional TESE [36]. Of course, an increased number of biopsies is always counterbalanced by a greater risk of damage to the vascularity of the testis, and so the surgeon must be constantly aware of this. For selection of the initial side, we prefer to start on the side with larger testicular volume or the side with the more advanced spermatogenic pattern seen on histology if a prior biopsy was done (with the most advanced being normal spermatogenesis followed by late maturation arrest, early maturation arrest and Sertoli-cell only pattern, in that order).

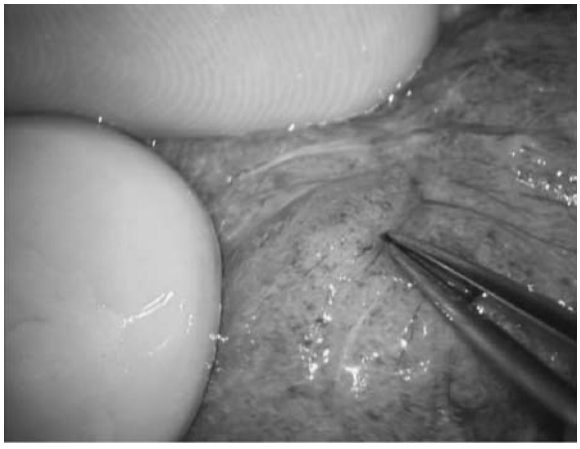

**Figure 17.2** Intraoperative picture of tubules more likely to harbor spermatogenesis, as indicated by forceps. See plate section for color version.

At Weill Cornell Medical College we have performed 81 mTESEs in 70 post-chemotherapy patients. These patients presented with a variety of malignancies, with the most common being Hodgkin's lymphoma, leukemia, testicular cancer and

**Table 17.1** Patients with underlying medical conditions treated with chemotherapy

| Medical condition | No. of patients ($n = 81$) | Percentage |
|---|---|---|
| Hodgkin's lymphoma | 29 | 35.8 |
| Testicular cancer | 13 | 16.0 |
| Leukemia (AML, ALL) | 13 | 16.0 |
| Non-Hodgkin's lymphoma | 12 | 14.8 |
| Sarcoma | 7 | 8.6 |
| Neuroblastoma | 3 | 3.7 |
| Medulloblastoma | 1 | 1.2 |
| Wilms' tumor | 1 | 1.2 |
| Mediastinal germ cell tumor | 1 | 1.2 |
| Nephrotic syndrome | 1 | 1.2 |

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

non-Hodgkin's lymphoma (Table 17.1). The mean number of years since chemotherapy was 18.6 years (range 1–34 years). The mean male age at mTESE was 35.3 years (range 22–53 years) and the mean female age was 32.5 years (range 21–43 years). Mean baseline FSH was 24.0 (range 4.2–62.7) and mean testosterone was 352 (range 64–814). Our sperm retrieval rate was 43.2% (35/81). Fertilization rate was 57.0% 192/337. Clinical pregnancies were defined as a heartbeat seen on transvaginal ultrasound 32 days after embryo transfer. The live birth rate was 42.9% (15/35) with 10 singleton deliveries and 5 twin deliveries. We noted a lower sperm retrieval rate with lymphoma 34% (14/41) than for testicular cancers 85% (11/13) [W. Hsiao *et al.*, unpublished data].

Damani *et al.* reviewed the University of California, San Francisco (UCSF) and Boston University experience in 2003 [37]. The series consisted of 23 patients who underwent chemotherapy for a number of reasons but mostly for testicular cancer. They either underwent conventional testicular sperm extraction or fine needle aspiration mapping and subsequent TESE. Sperm was successfully extracted in 65% (15/23) and a total of 26 cycles of ICSI were performed. The mean fertilization rate was 65% with a delivery/ongoing pregnancy rate of 20.8% in 11 couples. In a study of 12 patients post-chemotherapy, a multibiopsy approach TESE was undertaken and sperm successfully retrieved in 5/17. Eight ICSI cycles were performed with a fertilization rate per injected oocyte of 68%. There was only 1 live birth from 7 embryo transfers. [38].

## Donor sperm

### Third-party reproduction

Additional counseling is recommended for those patients who choose the assistance of third-party reproduction. There is universal agreement that the psychosocial, emotional and ethical complexities of donor conception require thorough exploration both for those donating and those receiving gametes [39–41]. In most clinics, a mental health professional (MHP) meets with prospective donors and recipients to explain the known psychological, social and legal implications of third party reproduction. It has been argued that the assistance of a MHP is essential to promote complete examination of the many dilemmas faced by those who receive gametes [42–44].

The MHP may need to help a patient address previously unresolved grief regarding the cancer diagnosis and treatment. When donor back-up treatment is being considered, it should be carefully explored prior to treatment and should not be a decision by default at the last minute (e.g. after poor TESE results and oocyte retrieval during an IVF cycle). Often times, a patient's hopes that viable sperm will be found may interfere with their ability to fully consider all aspects of a donor sperm back-up plan. While a patient may have grieved at the time of the cancer diagnosis, a diagnosis of infertility may reopen the grieving process by adding another dimension to the illness and may interfere with the desire to move forward with a donor.

Couples embarking on the path of third-party reproduction must mutually agree that their best alternative to genetic parenthood is the use of donated sperm or embryos. They must think about what it means to be a parent and how parenting a child who is not genetically connected to both of them may be different from parenting a child who is genetically connected to only one of them. Couples who feel strongly about their genetic lineage may view donor sperm/embryo as severing their ancestral ties. The ultimate loss of one's ability to create a child can create a powerful emotional crisis as well as feelings of sadness, anger and bereavement. There are several psychological losses to overcome in non-biological parenting: loss of biological posterity; loss of self-esteem and a sense of wholeness; loss of the ability to "give" one's partner a child; loss of the fantasized child that will embody the best of both parents; loss of a sense of control, health and well-being; and loss of the belief in the fairness of life. Resolution of these losses is best conceived as a process [45, 46].

In addition to thinking about how a third party, known or unknown, will affect their feelings about themselves and their relationship, most importantly, couples must think about their relationship with their potential child. Recipients must decide whether or not they plan to tell the potential child about how he or she was conceived and how much interaction, if any, they want the donor to have with the child. Couples often carry many fears and fantasies about gamete donation, including concerns that the donor will try to re-claim the offspring or that the child will wish to seek out his/her "real" mother or father. The man who is unable to use his own gametes may wonder if he is capable of loving "someone else's child." For some couples this process brings up thoughts and feelings about adultery, and they must work toward separating the act of sexual intimacy from the act of procreation. Others fear that the biological and genetic inequality of donor sperm may eventually threaten their relationship.

### Anonymous versus known sperm donation

There are three main types of gamete donation [47]. In anonymous donation, donors are typically selected from sperm banks and couples choose their donors from profiles with non-identifiable information such as physical characteristics, intelligence, academic history, professional background, hobbies, nationality, social history, religion and blood type. In other countries, it is sometimes the doctor that selects the donor according to phenotypological similarities with the male partner. The issue of an identified, known or interfamilial donor is another option for couples. When the donor is willing to disclose their identity, sometimes including meeting the parents (and possibly the child) in the future, the donor is referred to as a known or identified donor. A directed donor is a friend or relative of the intended parents who chooses to donate solely to that specific family.

The decision to use a known or unknown donor is only one of the many choices that affect all parties involved, including the potential offspring. Those who support anonymous sperm donation insist that anonymity is beneficial to the donor, the recipients and the donor offspring. Some couples strongly desire anonymous donation because they wish to maintain privacy about the donor decision, while others come to the process with additional losses because they do not know anyone who would be appropriate as a donor. Most couples who choose anonymous donation feel protected by the anonymity and feel that it creates a psychological barrier between them and the donor, enabling them to feel more secure as a family. Couples do not want the child to be confused about who his or her parents are or reject the non-genetic parent. They may also wish to conceal the donation from disapproving family members, especially those for cultures less accepting of sperm donation. Many couples worry that if their family knew about the donation, the child would not be loved or accepted in the same way as a full genetic child [48–50]. They typically feel that telling the child of his or her birth by sperm donation would subject the child to social or psychological disorders, which could be especially unsettling if the child wanted to find out more information about the donor but could not.

In recent years, a strong tendency in favor of non-anonymous sperm donation has emerged in Europe and Australia. Several countries have enacted laws or are taking into consideration permitting children to gain access to information about their genetic fathers once the child has reached maturity [51]. Proponents of non-anonymous sperm donation argue that human beings have a fundamental interest, and perhaps even a legal right, to know their biological origins. Not telling the child of his or her origins violates that child's autonomy. Proponents believe that disclosure is a key part of open and honest communication with children, which helps to avoid secrets in the family that can

damage family relationships and generate possible strain and anxieties.

Those who support known or non-anonymous donation feel more comfortable in having control over the source of the gametes as well as the knowledge of medical and social histories. Couples feel comforted by knowing firsthand the donor's personality, temperament and physical attributes, and may feel relieved with not having to deal with the social and relational confusion inherent in familial donation. Some choose a close friend, while others fear that sperm donation could jeopardize their relationship if something went wrong or if they did not conceive, or if the donor became attached to the child and viewed it as his. In some cases, known donors also give the child the option of knowing his or her genetic parent, which some donor recipient parents feel may help facilitate a more secure identity for their children.

Gamete donation has made it possible for participants to cross generational lines and has raised many complicated ethical issues. In 2003, the American Society for Reproductive Medicine (ASRM) issued an Ethics Committee Report on family members as gametes donors and surrogates [52]. While this report approved of many types of interfamilial gamete donations, it recommended a careful screening to ensure that the decision to donate gametes to a family member protected the autonomy of the donor. It also advised that semen donation should not be carried out in those situations in which the child would result from an incestuous (father-to-daughter donation) or consanguineous union (brother-to-sister donation). Furthermore, it recommends that family members (the extended family of the infertile couple) must be accepting of the resulting child. Interfamilial donations that the participants plan to keep secret from the larger family system should be carefully evaluated.

When an identified, known or interfamilial donor is being used there are specific psychological issues that need to be systematically addressed by the infertility counselor. It is necessary to assess the relationship between the participants to establish whether the reproductive plan is in the best interests of all of those involved, including the potential child. It is also important to obtain the history of the relationship between the donor and the recipient couples to ensure that there is no coercion or other "hidden" agendas. The nature of the relationship and boundaries between the potential child and the donor must be carefully examined and clearly defined for all parties. Should the relationship between the donor and the parents be strained at some time in the future, there is potential for traumatization of the potential child as well as other family members [49, 51]. In some cases, it is the counselor who needs to help one party say no to an uncomfortable request.

In child-to-parent donations, the counselor must address the imbalance of power and the inherent boundary violations that may leave the family system or the relationships vulnerable and at risk. Because most children feel indebted to their parents to some degree, these children are not truly free to say no to their parent's request in the same way they are free to say no to anyone else. In addition, many experts feel that the nature of the relationship may be violated as children are providing for their parents while these parents are still competent. The infertility counselor must also consider a son or daughter's relationship with the stepparent to make sure that there are no sexual overtones. In the case of a father donating to a son, some professionals feel more supportive, because the concept of a parent giving to their child is already built into the parent–child relationship [51, 53, 54].

Oftentimes couples may ask a brother to donate while others may ask another relative such as a cousin or a nephew. Brother-to-brother donations may appear to be ideal on many levels because of their similar genetic makeup and continuity of the bloodline, but it is only as good as the health of the relationships between the two siblings and their respective spouses. If couples choose a sibling to donate, old patterns of sibling rivalry may get stirred up. There are also many social and emotional entanglements that could occur in the family if their child for example, has an uncle who is considered his "genetic father" and a cousin who is his half-sibling. When third parties are involved in family building, especially when they are a family member, recipient parents may fear that their children are likely to form a stronger attachment to the donor than to them. When a family gamete cycle fails to result in pregnancy, all involved are extremely disappointed, and as family members search for an explanation, old family dynamics may be reenacted resulting in blame or feelings of guilt [53, 54].

It is critical for all of the parties involved in any type of known donation to have a clear understanding of boundaries and to think through scenarios that may challenge these arrangements in the future. All parties should be in agreement regarding disclosure to others as well as to the potential child. Ultimately, the donor should feel comfortable allowing the recipients

to make all decisions related to disclosure, the pregnancy and the upbringing of the potential child. The infertility counselor must help all of the parties involved explore their motivations, concerns, expectations, wants, hopes and fears regarding the process.

## Disclosure to children created with donor sperm

Whether or not children conceived using donated gametes should be told about their genetic origins and be able to access identifying information about the donor and/or be allowed to contact the donor are some of the most disputed ethical issues raised by the practice of assisted reproduction. The question of open disclosure versus secrecy is a complicated one, involving profound ethical, legal, religious and psychosocial issues. Some believe that it is not justifiable to keep such information secret, either because it is argued that children have an inherent human right to know about their genetic/gestational beginnings or because of concern about the effect of secrecy on family [55–57]. Others have argued that due to the limited available evidence about the risks and benefits of disclosure versus openness, neither view should be imposed on couples using donor gametes [58–60].

It is not surprising that a growing number of infertility counselors, professional organizations and government regulatory agencies recommend pretreatment counseling prior to donor sperm. Although the decision to use donated sperm is private, the issue of disclosure of this decision to others or to the resulting offspring often creates anxiety, questions and uncertainty for the recipient couple. Infertility counselors can provide a forum for patients to safely explore their thoughts and feelings about disclosure. There are arguments both for and against disclosure, and each couple should be allowed to decide with the help of infertility counselors which choice is best for them and their child(ren) within the context of these issues. Experts agree that the more the recipient couple feels comfortable and prepared for this parenting option, the more likely it is that they will be fulfilled as parents and will make decisions that are in the best interest of the child.

Haimes identifies three competing strategies for the management of genetic origins [61]. The first is full secrecy, on the grounds that there are no obvious benefits to be derived from disclosure and there is some risk of stigmatizing the offspring. The second is telling the truth about the means of conception and providing some basic information about the donor, but not revealing the donor's identity. The rationale here is to ensure that the offspring does not suffer from the effects of the family keeping such information secret. The third approach is telling the offspring as much as possible about the gamete donation and the donor, on the grounds that he or she will need information to develop a full sense of identity. Pennings proposed a fourth approach whereby donors may choose between anonymity or identification and recipients can opt for either an anonymous or identifiable donor [62].

Historically, parents have generally not been encouraged to tell others or their children that the family came to be through non-traditional means. The debate between disclosure and secrecy has its historical roots embedded in the traditions of sperm donation and adoption. The first case of artificial insemination using a donor was documented by William Pancoast, who claimed to have performed the procedure in secret. Thus began a trend of secrecy that has continued for over a 100 years for most couples choosing donor sperm. There are many reasons why donor sperm has been shrouded in secrecy. Male infertility is often associated with impotence or a lack of virility and sexual functioning, and thus carries with it a shameful stigma that is seldom discussed openly among health professionals [51]. Donor insemination (DI) itself has been associated with masturbation and the involvement of a second male has been suggestive of an extramarital affair.

There are many reasons why a couple may choose not to tell family, friends and/or the child. Shame often fuels a non-disclosure stance. Unfortunately, shame is a byproduct of secrecy, which only increases the couple's feelings of inadequacy and may decrease their ability to form close relationships with the child. Couples may hold fast to the illusion that if they do not tell, they will remain protected from the sadness of not having a family by traditional means. For some religions, the use of donor sperm is discouraged since it is viewed as having a third party in marriage and has been compared to sexual infidelity. However, families who protect secrets develop a complicated system of interpersonal relationships with taboo-like undercurrents that children often pick up on. This system of secrecy can inadvertently promote family estrangement and create unhealthy alliances between those who know and those who do not know and may also undermine the trust that is vital to a healthy parent–child relationship.

When parents choose not to tell their children, they often live in fear that they may find out about their origins and that the bond of trust that children have with their parents will be threatened.

This trend toward secrecy appears to be slowly changing as people have come to recognize the hazards of secrecy and the needs and rights of the offspring to access information about their biological origins. The psychological wishes of sperm donors and their attitudes toward non-anonymity and disclosure are increasingly given consideration. Recent findings showed an increase in donor programs that offer open-identity between donors and offspring [63, 64].

In addition, legislation, professional guidelines and MHPs in several countries have supported open information sharing [51]. In 2004, the ASRM published recommendations that encouraged disclosing the use of donated gametes to offspring [65]. Legislators' responses to this debate vary. In some countries, such as France, Denmark and Spain, gamete donation only occurs with reciprocal anonymity between donors and recipients. In others, such as the UK, Austria, New Zealand, Sweden and some states in Australia, irrespective of whether the donor is anonymous or known, it is a legal requirement that identifying information about the donor is recorded to enable a child born as a result of a donor procedure to access it in the future [66]. Policy changes in other countries have varied. Although there is no agreement within the European Union regarding access to identifiable information about the donor, from 2006 on, all member countries are required to document information about gamete donors for a minimum of 30 years [51]. There are still some countries, such as Italy, where ART legislation was introduced in 2004, where treatment using donated gametes is banned [67].

Nevertheless, as a result of different values attached to family, marriage and the child's well-being, most families do not disclose the use of donor insemination to children [51, 68]. Reasons cited for non-disclosure include the desire to protect the child from either the distress and stigma of finding out that the father is not the genitor; the impossibility of accessing information about the donor; to protect the father from being rejected by the child; the belief that disclosure is unnecessary; concerns that family relationships may be damaged as a result of disclosure; the lack of educational material/resources; and lack of support and guidance on how to tell the child [66, 69–71]. Couples also report the desire to "normalize" the situation, both so that the child does not feel different and the husband does not feel embarrassed, and to allow the parents to feel that they are an ordinary family.

Religion and culture often influence attitudes about the use of donor sperm. Donor insemination remains a morally questionable treatment option in many countries. Using donor gametes is forbidden in some religions. In Islam, only a married couple's eggs and sperm may be used in procedures to treat infertility because religious law dictates the preservation of the genetic line. The Vatican does not endorse any assisted reproductive techniques, even insemination with the husband's own sperm [72]. Some Catholic bioethicists have supported assisted reproductive techniques if no other alternatives exist to allow a couple to have children but still do not find use of donor sperm acceptable [73].

More often, couples are being encouraged to provide genetic disclosure to offspring. This emerging trend of more openness may reflect legislative and cultural changes as well as the impact of counseling and the advent of guidance materials parents can use for talking to their children [51]. The main reasons that couples choose to disclose include: the children had a right to this information; the parents wanted to avoid the burden of secrecy and the risk of disclosure by somebody else or accidental discovery; and the parents believed that technical advances in genetics could result in a genetic mismatch discovered by the offspring, again resulting in "inadvertent disclosure" [74, 75].

If couples choose to disclose the information to their children, they should also understand the distinctions between openness and privacy. Couples still have the right to discern who will know, how they will find out and when they will share the information. Given the stigmatizing nature of using donor sperm and male-factor infertility, the couple needs to be in agreement about with whom they will share information regarding infertility treatment, diagnosis and their use of donor sperm. If they do not intend to talk to their future child about his or her donor sperm origins, they must attempt to ensure that their confidants will not disclose this to the child accidently nor discuss the issue of donor sperm with others.

Information sharing will not only be an individual decision based on personal preference but will also depend on cultural and religious factors, such as the degree of acceptance of donor sperm as well as legal practices regarding access to information and

donor identity. Research on how parents of donor offspring make decisions about disclosure reveals that even when couples are initially opposed to disclosing to their offspring, most ultimately come to a united disclosure decision either "intuitively," or after discussions influenced by the couples' local sociopolitical environment, professional opinion, counseling, religious and cultural background, family relationships and personal, psychological and ethical beliefs [76]. Moreover, when gamete donation is used because of a parent's history of cancer, telling the child may provide reassurance about his or her own lifetime cancer risk.

Age of disclosure appears to be important in determining donor offspring's feelings about their donor conception. It seems that it is less detrimental for children to be told about their donor conception at an early age [77]. Those told later in life report more negative feelings regarding their donor conception than those told earlier. Offspring from heterosexual couple families are more likely to feel angry at being lied to by their mothers than by their fathers. The most common feeling toward fathers was "sympathetic." While the research in this area is somewhat limited, the largest prospective follow-up of donor insemination children to date documents normal social and psychological adjustment of children and families as the cohort reached 12 years, despite the decision of almost all parents to maintain secrecy with their offspring [78]. Golombok *et al*. compared natural, adopted in infancy, donor sperm and IVF children up to the age of 12 years and found that parents who conceived without difficulty had higher levels of parenting stress than the other groups [79]. No significant differences were found between types of families in the children's behavior, adjustment at home or school or feelings toward their mothers or fathers. In assessing the quality of the parent–child relationship, Golombok also found that IVF, DI and adoptive mothers had higher levels of warmth and emotional involvement with their children [79]. In vitro fertilization or DI fathers were rated as displaying more warmth toward their children than natural conception or adoptive fathers.

Overall, cancer survivors tend to view parenthood in a positive manner and feel that their experience with cancer would make them better parents [4]. Although cancer survivors can become parents through options such as adoption and third party reproduction [80], most prefer to have genetic offspring [5, 81], even if they have concerns about abnormalities that could result if the parent conceived before cancer treatment [82] or anxiety about their own longevity or their child's risk of cancer [4]. Research is needed to address the psychological sequelae of a cancer diagnosis and treatment, and the contribution that fertility preservation may make on quality of life post-treatment. More information available to oncologists, reproductive endocrinologists, urologists and MHPs working with cancer patients, could be beneficial in facilitating the psychological adaptation and quality of life of cancer survivors, and helping them make difficult and critical decisions regarding future fertility and family building.

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Chapter 18

# Use of GnRH agonists for prevention of chemotherapy-induced gonadotoxicity

Susannah C. Copland and Megan Clowse

The use of gonadotropin-releasing hormone agonists (GnRHa) for prevention of chemotherapy-induced gonadotoxicity remains controversial [1, 2]. Ovarian suppression to prevent oocyte loss during chemotherapy was proposed based upon the observation that non-cycling cells appear more resistant to cytotoxicity and that pre-pubertal girls resume menstruation after cancer treatment more often then post-pubertal girls [3]. Studies in rats and primates provide some support for the use of GnRHa for ovarian protection [4, 5]. Studies in humans that suggest benefit, however, have been predominantly observational with historic controls. The efficacy of GnRHa to protect the ovary during chemotherapy has yet to be proven in adequate randomized control trials; however, use of this treatment strategy with the goal of preventing premature ovarian failure is becoming more common. The 2006 American Society of Clinical Oncologists consensus statement on fertility preservation recommendations for cancer patients emphasized the need for safety and effectiveness data and recommended that patients considering the option of GnRHa enroll in clinical trials [6]. Several randomized trials are in progress, both for patients utilizing gonadotoxic agents for cancer and for rheumatologic conditions; however, the results of these trials have yet to be published. This chapter summarizes the current evidence and debate for and against the use of GnRHa during chemotherapy.

## Natural GnRH and ovarian steroidogenesis

Natural human GnRH is released from the hypothalamus in a pulsatile fashion to stimulate gonadotropin release from the pituitary gland. The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), dictate the ovulatory cycle and consequent ovarian steroid and peptide hormone production. The ovarian hormones provide feedback to the hypothalamus and pituitary to modulate gonadotropin release. The complex interplay of hypothalamic pituitary ovarian signaling facilitates the three distinct phases of the menstrual cycle: the follicular phase, during which the dominant follicle is selected, estradiol levels rise and the endometrium proliferates; ovulation, during which the dominant follicle releases the oocyte and becomes the corpus luteum; and the luteal phase, during which the corpus luteum produces estradiol and progesterone, the endometrium becomes secretory to facilitate implantation of a potential pregnancy, until eventual menstruation with corpus luteum involution if pregnancy does not occur (Figure 18.1).

## Mechanism of GnRHa action

The GnRHa suppress the hypothalamic–pituitary–ovarian axis, and thereby suppress ovarian ovulatory follicle development and resulting steroidogenesis. Endogenous GnRH is a 10 amino-acid peptide. Sustained release synthetic GnRHa alter 1 or 2 of the 10 amino acids facilitating greater binding affinity and slower degradation. The GnRHa bind to GnRH receptors on the pituitary gland for longer periods than natural human GnRH. After exposure to GnRHa, the pituitary gonadotropes increase the GnRH receptor number, which increases production and release of gonadotropins, a phenomenon commonly known as the flare response. With prolonged gonadotrope

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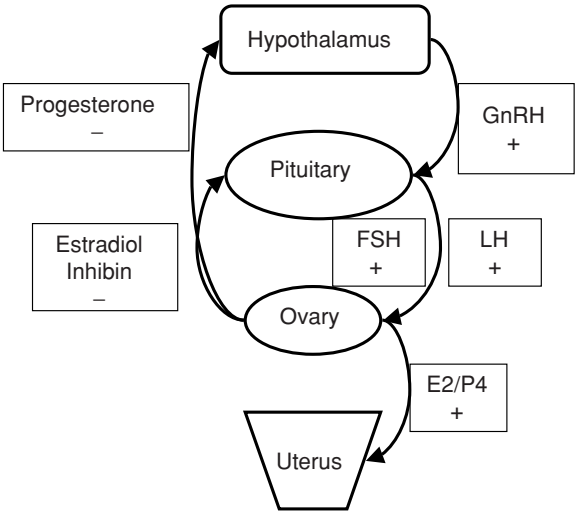


**Figure 18.1** Hypothalamic pituitary ovarian signaling. E2, estradiol; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; P4, progesterone.

exposure to GnRHa, the ligand–receptor complexes are internalized and degraded; this results in a decrease in GnRH receptor number, and concordant desensitization of the pituitary to further GnRH stimulation. Therefore, after the initial release of FSH and LH, further gonadotropin release is prevented. Without pituitary gonadotropin stimulation, the ovulatory ovarian cycle ceases, resulting in decreased estradiol and progesterone production to postmenopausal levels and amenorrhea [7].

## Clinical use of GnRHa

### The flare response

With the initial dose of GnRHa, the pituitary gland releases endogenous gonadotropins. This initial FSH release, or flare, can stimulate the ovary. After continued GnRHa exposure, further FSH release is prevented. The amount of FSH released is greatest in the first few days following the first dose of GnRHa. By 1–2 weeks, low gonadotropin levels are achieved. The extent of the flare response to GnRHa depends upon the time of administration with a patient's menstrual cycle. During the follicular phase of the menstrual cycle, ovarian steroids are low providing little endogenous suppression of the pituitary and a larger flare response. During the luteal phase of the menstrual cycle, ovarian steroids produced by the corpus luteum provide endogenous suppression of the pituitary and decrease the extent of the flare. Women with polycystic ovaries and increased ovarian stroma exhibit an exaggerated flare when compared to normal controls [8].

Theoretically, ovarian stimulation from the flare response may make the ovary more vulnerable to insult from chemotherapy. Indeed, chemotherapy itself may stimulate the ovary. Letterie reported a greater number of medium and large follicles in cytoxan-exposed rats than control; ovarian suppression with GnRHa or oral contraceptive pills was not protective [9]. To mitigate any potential harm from the flare response, administration of GnRHa 2 weeks prior to chemotherapy start has been proposed.

Others have proposed using a GnRH antagonist (GnRHant) concordant with the initial dose of GnRHa to decrease the intensity and duration of the flare. The GnRHants competitively inhibit GnRH binding to the pituitary GnRH receptors. A depot formulation is not approved for use in human; however, the short-acting GnRHant can be administered every 3 days during the initial days of GnRHa-induced flare to mitigate the gonadotropin release. Coadministration of GnRHant with GnRHa decreased the duration of the flare response in Roth *et al.*'s pilot study of children with precocious puberty or short stature [10]. For the added expense of GnRHant to be worthwhile, the decrease in flare response would need to add sufficient benefit to outweigh increased cost and not add any additional harm. Danforth *et al.*'s report of GnRHant depletion of primordial follicles in mouse raises concern for potential harm [11]. Where GnRHa significantly decreased the primordial follicle loss associated with cytoxan administration and had no direct effect on primordial follicle count in the absence of cytoxan, GnRHant did not protect the ovary from cytoxan. Indeed, both systemic and direct ovarian GnRHant administration resulted in significant decreases in primordial follicle count even at doses insufficient to decrease uterine weight, a surrogate for gonadotropin suppression [11]. Danforth *et al.*'s surprising report of a negative effect of GnRHant on primordial follicle number is in direct contrast to Meirow *et al.*'s prior report of GnRHant protecting against cytoxan-induced follicular decline in mouse [12]. Meirow *et al.* using a different strain of mouse and a different dose and frequency of GnRHant, reported no direct effect of GnRHant on follicle number compared to saline and a protective effect of decreased primordial follicle loss when used concurrently with cytoxan [12]. These discrepant findings require further study to

delineate the effect of GnRHant on primordial follicles with and without chemotherapy [13].

## Dose

Gonadotropin-releasing hormone analogues can be administered in many formulations with different durations of action. In human studies, the dose of GnRHa has been evaluated by the ability to suppress ovarian and testicular steroid hormone production. Whether this dose is sufficient for ovarian protection depends on the potential mechanism of protective effect. Some propose that GnRHa may protect the ovary by inducing a pre-pubertal state. Whether dose and formulation of GnRHa influences the level of pituitary suppression at the end of the labeled depot period has been examined in children using GnRHa to treat central precocious puberty. With each repeat dose there is the possibility of an increase in FSH release if the pituitary suppression from downregulation has decreased. The rise in FSH and LH after repeat dosing was higher with the lower dose, longer depot duration formulations (Depot–leuprolide 3.75 mg – 1 month, 11.25 mg – 3 months) than the higher dose, shorter depot duration formulation tested (Depot–leuprolide 7.5 mg – 1 month). While a greater magnitude of pituitary responsiveness was seen with the lower dose, longer duration formulations, gonadal steroid production and pubertal stage did not change [14]. Mohamed *et al.* also documented a statistically significant increase in pituitary response to GnRH administration after 2 months of downregulation with goserelin 3.6 mg subcutaneous pellet in 21 patients with endometriosis [15]. Changes in ovarian steroids were not reported; therefore, it is not known whether the pituitary responsiveness at the end of the depot period is clinically significant. Pending additional studies with biochemical and clinical outcomes, the relevance of these fluctuations of FSH at the time of repeat dosing is unknown.

The influence of obesity upon GnRHa effectiveness has been questioned. Obese men using GnRHa for treatment of prostate cancer exhibit higher testosterone levels than men with normal body mass indexes [16]. The GnRHa active peptide is hydrophilic and is therefore most influenced by the constant volume of the extracellular fluid compartment. The synthetic polymer linked to the GnRHa, however, is lipophilic. Agarwal therefore postulated that adiposity may influence the rate of enzymatic breakdown of the lipophilic polymer; more rapid breakdown would empty the depot site sooner resulting in a decrease in gonadal suppression before the labeled period [17]. Further study is needed to determine whether obese patients would benefit from higher doses or different formulations of GnRHa.

## Side effects

The most common side effects of GnRH analogues are related to the subsequent estrogen deprivation. Vasomotor symptoms, hot flushes, night sweats, vaginal dryness and headaches can occur. With longer duration of administration and the subsequent estrogen deprivation, bone loss can occur. Side effects can be mitigated by hormone replacement. A combination estrogen–progestin product such as a birth control pill will protect bone and prevent vasomotor symptoms. If estrogen exposure is contraindicated, a progestin-only formulation can be of benefit.

## GnRHa and ovarian reserve

The most established effect of GnRHa is reduction of gonadal steroid production via cessation of the ovulatory ovarian cycle. The ovulatory cohort of oocytes, however, has reached a point in development where they will either ovulate or undergo degeneration [18]. Therefore, despite the established effect of GnRHa to prevent the ovulatory cohort from ovulating, this cohort of oocytes will still be lost to atresia. Dormant primordial follicles are the largest contributor to the total oocyte pool. For GnRH analogues to protect an ovary from chemotherapy, they need to exert effects beyond preventing ovulation of the ovulatory cohort and protect total oocyte number or ovarian reserve.

Ovarian reserve reflects the number and quality of oocytes remaining in the ovary. Women are born with their total oocyte complement. Though women start life with over 1 million oocytes, egg number declines with age. Several hundred thousand remain at puberty. Only 300–400 will ever be released through ovulation; the rest go through atresia.

Before puberty, oocyte number decreases via atresia, but without the appropriate hypothalamic–pituitary signaling, there is no ovulation. After puberty, hypothalamic–pituitary signaling results in pituitary–gonadotropin stimulation to the ovary. Each month a group of oocyte-containing follicles gain the ability to compete to ovulate. If the hypothalamic–pituitary signals are present, these

follicles will compete, the oocyte from the dominant follicle will be released through ovulation, and the rest of the group will undergo atresia [18]. Surgery, chemotherapy and radiation cause loss in ovarian reserve that is superimposed upon the loss due to natural reproductive aging.

The studies of GnRHa during chemotherapy enumerated below have focused on menstrual outcomes. While menses is a surrogate marker for ovarian function, menstrual function does not completely predict the clinical outcomes of most interest: steroidogenesis and ovulation resulting in live birth. The average age of menopause is 51 years; the average age of last baby born in societies not using contraception is 41 years. Therefore there are 10 years of menses before menopause that are not on average resulting in birth [19]. Future studies need to include the clinical endpoints necessary for patient counseling including: live birth, hormone production, including any changes to bone density and age of eventual menopause.

## Clinical data about GnRHa and ovarian preservation

Cotherapy of a GnRHa during chemotherapy has been under investigation since the mid 1990s. Observational studies, without a control group, show some promising results (Table 18.1 [20–26]). In each of these studies the definition of maintained ovarian function was resumption of menses in the months following chemotherapy. Some studies also measured FSH and estradiol; these were not measured on cycle day 3, but randomly at the time of follow-up. For women undergoing multiple doses of chemotherapy with or without radiation, the rate of menses return ranged from 80 to 97%. Several pregnancies were reported in these studies, but none reported the number of women who tried unsuccessfully to conceive.

Age at the time of chemotherapy appears to be an important determinant of the success of this therapy. In one study of women with early breast cancer, 97% of women under the age of 40 resumed menstruation compared to 42% over the age of 40. In addition, menses were slower to return following chemotherapy with advancing age [20].

The total dose of chemotherapy was also important. In a study of women with lupus nephritis, only 2 out of 25 women had ovarian failure, both were over the age of 35 and received a second cycle of cyclophosphamide for relapse [26]. In one small report of 5 women, the only woman without a resumption of menses received a more aggressive course of chemotherapy over 34 weeks for stage IVA Hodgkin's disease, compared to 16–24 weeks for stage IIA or B Hodgkin's disease in the other women [25].

The marrow-ablative regimen required for stem cell transplant resulted in ovarian failure in all 30 women despite prior treatment with leuprolide 3.75 mg [23]. Most of the women received busulfan 4 mg/kg for 4 days and cyclophosphamide 60 mg/kg for 2 days; other women received variants that included anti-thymocyte globulin (ATG) or melphalan. Only one of the women had menstrual bleeding during the time of thrombocytopenia following chemotherapy. Hormonal status was followed over the following year with FSH consistently elevated (median 35 mIU/ml, range 30–80 mIU/ml) and estradiol low (median 15 pg/ml, range 10–25 pg/ml).

Bone density declined during GnRHa therapy in several studies. In one study of 18 women, the hip and spine T scores declined by an estimated 0.4 points during the 6 months of cyclophosphamide, high dose prednisone and leuprolide. In the following 6 months, the BMD increased, though not back to baseline, in the women who had a resumption of menses. It did not increase in the women with premature ovarian failure [21]. Another study showed both decreases and increases in bone density during therapy with some women with normal density developing osteopenia, several improving from osteoporosis or osteopenia, and many remaining unchanged [27]. Another reported a significant decline within just 3 months of goserelin therapy, with median T scores falling from −1.50 to −2.40. Bone density measurements following the resumption of menses were not reported in this study [20].

Some studies included "add-back" estrogen therapy to diminish the side effects of GnRHa therapy. In the only study that reported side effects, women did not receive add-back therapy and 97% had hot flashes, 90% headaches, sweating or mood changes, and 59% reported vaginal dryness [20]. It is not clear how effectively estrogen therapy might relieve these symptoms. Some women, particularly those with breast cancer and lupus, are not candidates for estrogen therapy.

### Studies with a control group

While much of the data from observational studies is encouraging, it does not document the true benefit of

**Table 18.1** Clinical data from studies of gonadotropin-releasing hormone agonist (GnRHa) co-therapy without a control group

| | GnRHa | | | | |
|---|---|---|---|---|---|
| **Author (*Location of study, year*)** | ***N*** | **No. with ovarian function** | **Percentage with ovarian function** | **Definition of ovarian function** | **Disease, chemotherapy** |
| Del Mastro *et al.* [20] *(Italy,* 2006) | 29 | 27<br><br>21<br>24/25 | 97<br><br>72<br>96 | Either menses or FSH≤40<br><br>Menses<br>FSH ≤40 | Early breast cancer Goserelin 3.6 mg 1 week prior, then q 4 weeks |
| Perez Pampin *et al.* [21] *(Spain* 2006) | 18 | 15 | 83 | Menses | Lupus: intravenous monthly pulses of CYC Lupron 3.75 mg |
| Castelo-Branco *et al.* [22] *(Spain,* 2009) | 45 | 40 | 89 | Menses | 30 Hodgkin's, 15 non-Hodgkin's lymphoma: chemo and XRT<br>Triptorelin 3.75 mg 1–2 weeks prechemo then q 4 weeks **plus** tibolone or OCP |
| Chiusolo *et al.* [23] *(Italy 1998)* | 30 | 0 | 0 | Menses and FSH | Stem cell transplant: 11 AML, 7 Hodgkin's, 7 non-Hodgkins, 1 CML, 3 ALL, 1 MM<br>Leuprolide 3.75 mg >30 days prior to chemo then 1 dose 28 days after 1st dose |
| Potolog-Nahari *et al.* [24] *(Israel,* 2007) | 9 | 8 | 89 | Menses | 3 Hodgkin's, 1 non-Hodgkin's, 3 B cell lymphoma, 1 breast cancer, 1 Wegener's granulomatosis<br>Goserelin 3.6 mg 1–2 weeks prior then q 4 weeks |
| Franke *et al.* [25] *(the Netherlands,* 2005) | 5 | 4 | 80 | Menses | Hodgkin' s disease: chemo and XRT<br>Goserelin 3.6 mg 1–2 weeks prior then q 4 weeks |
| Dooley *et al.* [26] (*USA, 2000*) | 25 | 23 | 92 | Menses | Lupus nephritis: intravenous pulse CYC Leuprolide 3.75 mg q 4 weeks |

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; chemo, chemotherapy; CML, chronic myeloid leukemia; CYC, cyclophosphamide; FSH, follicle stimulating hormone; MM, multiple myeloma; OCP, oral contraceptive pill; XRT, radiation therapy.

GnRHa. To do this, randomized controlled trials of this therapy are needed. At this time, two randomized trials have been published, in addition to nine other studies that compared women treated with GnRHa co-therapy to a control group. All but one of these studies agree that GnRHa therapy increases the likelihood of resuming menstruation following chemotherapy. We completed a meta-analysis of the first 9 published studies and found that GnRHa co-therapy is associated with a 68% increase in the rate of preserved ovarian function [28]. This study excluded the two most recent studies, both of which corroborate this finding [29, 30].

The first published study did not demonstrate benefit, with 4 of 8 women with GnRHa co-therapy resuming menses versus 6 of 9 women without this treatment [31]. This study is the only one to use intranasal buserelin three times per day to maintain ovarian suppression. Unfortunately, this dosing was likely inadequate to fully suppress the pituitary–ovarian axis. A study of intranasal buserelin for the therapy of breast cancer demonstrated that method of GnRHa therapy was inadequate for full ovarian suppression [32].

All subsequent studies have shown that co-therapy with GnRHa helps to retain ovarian function during chemotherapy.

A study that was conducted in the early 1990s but reported in 2009 found somewhat contradictory results [30]. Women with breast cancer were treated with cyclophosphamide, methotrexate, fluorouracil (CMF) chemotherapy if lymph nodes were positive and were divided into 4 groups for 2 years of endocrine therapy: control, goserelin (3.6 mg q 4 weeks), tamoxifen (40 mg po qd) or goserelin plus tamoxifen. The women who received goserelin alone during CMF treatment resumed menstruation 36% (8/22) of the time. This compares to 10% of the control group, 13% of the tamoxifen group and 7% of the tamoxifen plus goserelin group. Why the goserelin group had more menstruation resumption than the combined tamoxifen plus goserelin group is unclear, but the authors speculate that the estrogenic properties of the tamoxifen may have diminished the effects of the GnRHa. It should also be noted that the first dose of goserelin was given simultaneously with the first chemotherapy dose, likely leading to a greater level of ovarian damage as the chemotherapy affected the ovary during a time of increased activity.

Two recent randomized trials have shown benefit from GnRHa co-therapy. Badawy *et al.* randomized 80 women with breast cancer to co-therapy with goserelin 3.6 mg every 4 weeks [29]. Of the women who received the co-therapy, 90% resumed menstruation and 69% resumed spontaneous ovulation 8 months following chemotherapy, compared to 33% menstruating and 26% ovulating after chemotherapy alone. Guiseppe Loverro and his team treated 29 women with Hodgkin's disease to either triptorelin or no co-therapy [33]. All of the women with co-therapy resumed menstruation compared to 47% of those without it (Figure 18.2 and Table 18.2 [34–39]).

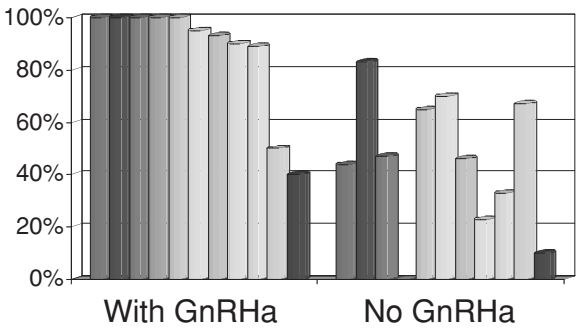


**Figure 18.2** Percentage of women with a return of ovarian function following chemotherapy. Each study is a different color. GnRHa, gonadotropin-releasing hormone agonist. See plate section for color version.

## Putative mechanisms of ovarian impact

The mechanisms by which GnRH analogues could affect ovarian reserve are the subject of much debate. Putative mechanisms include: changes to ovarian stromal blood flow; direct effect of GnRH; indirect effect through suppression of FSH; and GnRH-dependent immunological changes.

## Ovarian stromal blood flow

If prolonged GnRHa administration decreases ovarian blood flow, then less chemotherapy may reach the ovary. Existing studies of ovarian blood flow after pituitary downregulation, however, report conflicting results. In Engmann *et al.*'s study of 99 women undergoing pituitary down regulation for in vitro fertilization (IVF), ovarian stromal artery peak systolic velocity significantly decreased after 2–3 weeks of daily subcutaneous buserelin [40]. Dada *et al.* also showed decreases in utero-ovarian blood flow after pituitary downregulation with both subcutaneous buserelin and intranasal nafarelin [41]. In contrast, Jarvela *et al.*'s 2003 study of 40 women undergoing pituitary

**Table 18.2** Clinical data from controlled studies of gonadotropin-releasing hormone agonist (GnRHa) co-therapy

| | GnRHa | | | No GnRHa | | |
|---|---|---|---|---|---|---|
| **Author *(Location of study, year)*** | ***N*** | **No. with ovarian function** | **Percentage with ovarian function** | ***N*** | **No. with ovarian function** | **Percentage with ovarian function** |
| Blumenfeld and Eckman [34] *(Israel,* 2005) | 75 | 70 | 93 | 82 | 38 | 46 |
| Blumenfeld *et al.* [35] *(Israel,* 2000) | 8 | 8 | 100 | 9 | 4 | 44 |
| Castelo-Branco *et al.* [22] *(Spain,* 2007) | 30 | 27 | 90 | 26 | 6 | 23 |
| Dann *et al.* [36] *Israel,* 2005) | 7 | 7 | 100 | 6 | 5 | 83 |
| Loverro *et al.* [33] *(Italy, 2007)* | 14 | 14 | 100 | 15 | 7 | 47 |
| Petri *et al.* [37] *(USA, 2004)* | 4 | 4 | 100 | 17 | 11 | 65 |
| Somers *et al.* [38] *(USA, 2005)* | 20 | 19 | 95% | 20 | 14 | 70 |
| Waxman *et al.* [31] *(UK, 1987)* | 8 | 4 | 50 | 9 | 6 | 67 |
| Pereya *et al.* [39] *(Argentina, 2001)* | 12 | 12 | 100 | 4 | 0 | 0 |
| Sverrisdottir *et al.* [30] *(Iceland,* 2009) | 22 | 8 | 36 | 72 | 7 | 10 |
| Badawy *et al.* [29] *(Egypt, 2009)* | 39 | 35 | 89 | 39 | 18 | 33 |

downregulation for IVF found no difference in three-dimensional power Doppler vascularity color indices after 2 weeks of daily subcutaneous buserelin [42]. Yu Ng *et al.*'s report of 85 women undergoing luteal pituitary downregulation for IVF after a median of 10 days of intranasal buserelin also found no difference in three-dimensional power Doppler flow indices [43]. The goal of GnRHa administration in IVF is to prevent ovulation without overly suppressing ovarian response. In the context of these IVF studies using lower daily GnRHa dosing, GnRHa have not been reproducibly proven to decrease ovarian blood flow; the potential effect of higher doses of GnRHa from depot formulations on ovarian stromal blood flow is as yet unknown.

## Direct and indirect effects of GnRH or FSH

Direct effects of GnRHa or FSH on ovarian tissue may influence ovarian response to chemotherapy. Direct effects would assume presence of receptors upon the primordial follicle, which has not yet been described. Indirect effects via changes in detoxifying enzymes or immunological responses would not require receptor presence upon primordial follicles, and have therefore also been proposed.

## GnRH expression in the ovary

In Choi *et al.*'s immunohistochemical study of pre-menopausal ovaries, GnRH-I, GnRH-II and GnRH receptor were identified in the granulosa cell layer of pre-ovulatory follicles, the corpus luteum and the ovarian surface epithelium, but not the primordial to early antral follicles which represent the largest portion of the ovarian reserve [44]. The absence of GnRH receptors on the primordial follicles makes a direct GnRHa effect at the primordial follicle level less likely.

Pre-treatment of granulosa cell cultures from pre-ovulatory follicles with the GnRHa buserelin

prior to doxorubicin exposure preserved subsequent granulosa cell estradiol production in response to FSH-containing media. In the absence of GnRHa pre-treatment, doxorubicin induced a concentration-dependent inhibition of estradiol secretion. Similar results were seen with leuprolide. The granulosa cells for the study were obtained, however, from aspiration of pre-ovulatory follicles where GnRH receptors have been identified. This evidence may not reflect the behavior of granulosa cells from primordial and pre-antral follicles, which lack GnRH receptors and are the majority of the follicle pool [45].

Emons *et al.*'s phase II clinical trial of the GnRHant cetrorelix in the treatment of plantinum-resistant ovarian or Müllerian cancer, reviews three signaling mechanisms by which GnRHa and GnRHant may exhibit dose-dependent inhibition in the majority of ovarian and endometrial cancers [46]. The ligand-bound GnRH receptor couples to G-protein αi activating: (1) inhibition of mitogenic signaling, with reduced epidermal growth factor (EGF) -induced cell proliferation; (2) nuclear factor kappa B anti-apoptotic mechanisms; (3) cell cycle arrest in $G_0/G_1$ phase via activation of Jun kinase pathway and induction of AP-1. These inhibitory effects of GnRHa and GnRHant upon cancer cell proliferation raise concern for prevention of intended chemotherapy-induced apoptosis and unintentional protection of cancer cells if GnRHa or GnRHant are co-administered with the chemotherapeutic regimen [46]. Data from the LH-releasing hormone (LHRH) -agonists in the Early Breast Cancer Overview group is, however, reassuring. In Cuzick *et al.*'s meta-analysis of premenopausal women with hormone-receptor positive breast cancer enrolled in randomized controlled trials including a GnRHa, addition of GnRHa to tamoxifen, chemotherapy or both ($n = 3754$) reduced the hazard rate for recurrence by 12.7% (95% CI 2.4–21.9) and for death after recurrence by 15.1% (95% CI 1.8–26.7) [47]. The addition of both GnRHa and tamoxifen to chemotherapy ($n = 1210$) reduced the hazard rate for recurrence by 26.7% (95% CI 12.3–38.7) and for death after recurrence by 24.4% (95% CI 6.4–39.0) [47]. This survival benefit with addition of GnRHa to chemotherapy in hormone-receptor positive breast cancer suggests that, even if the described inhibitory effects of GnRHa upon cancer cell proliferation are involved, the survival benefits in the context of hormone-reponsive breast cancer outweigh the theoretical risks.

## FSH expression in the ovary

An indirect effect of GnRHa on the ovary via suppression of FSH has also been discussed. While FSH is clearly involved in cyclic recruitment of the ovulatory cohort, a role for FSH in the dormant cohort of primordial follicles has yet to be shown. Oktay *et al.* performed real-time polymerase chain reaction (RT-PCR) for FSH-receptor mRNA on ovarian biopsies from 11 women at cesarian: none of the primordial follicles; 33% of the primary and 2 layer follicles; and 100% of multilayer follicle expressed FSH receptor mRNA [48]. Patsoula *et al.* identified FSH receptor mRNA in surplus oocytes from IVF [49]. Pre-ovulatory oocytes collected during IVF, however, cannot be assumed to reflect the expression of primordial follicles [49].

## Indirect effects of FSH suppression

While a direct effect of FSH on primordial follicles is less likely given their absence of FSH receptors, an FSH-mediated effect in the ovary is still plausible. Toft *et al.* documented a FSH effect on detoxifying enzymes glutathione transferase in the rat liver and ovary [50]. While the glutathione transferase isoenzymes expression vary in response to rat sexual maturation and exogenous gonadotropin administration, clinical outcomes were not assessed [50]. Oktay *et al.* have postulated that changes detoxifying enzymes theoretically may cause harm to the ovary if the gonadotoxicity of chemotherapy were subsequently increased [51].

For GnRHa to be of benefit to fertility preservation, they would likely need to spare both oocyte quantity and quality. Familiari *et al.* evaluated follicle number and structure in ovarian biopsies from women with Hodgkin's disease before multidrug chemotherapy or depot-medroxyprogesterone acetate (depot-MPA), before chemotherapy with depot-MPA and after 4–5 menstrual cycles after chemotherapy with depot-MPA ovarian suppression [52]. While coadministration of depot-MPA appeared to spare follicle numbers when compared with numbers reported from prior studies, a higher proportion of follicles from biopsies obtained after chemotherapy were undergoing atresia [52]. The long-term significance of this increased proportion of atretic follicles, and whether ovarian suppression with GnRHa would yield similar ultrastructural findings, requires further study with longer follow-up of clinical outcomes.

## Immunological effect of GnRHa

Whether the immunological effects of GnRHa could influence the gonadotoxic effect of chemotherapy has yet to receive much attention. Umathe *et al*. report that GnRHa prevents stress-induced immunosuppression in mice [53]. Leuprolide subcutaneously administered 30 min prior to restraint stress prevented the decrease in thymus weight, leukocyte count and humoral and cell-mediated immune response markers seen in controls. Prior intracerebroventricular administration of GnRHant and castration did not change results. Therefore, this immunological effect appears peripheral, independent of the hypothalamic effect [53]. In their report of GnRHa use prior to stem cell transplant, Sutherland *et al*. reported that goserelin 3 weeks prior to and after hematopoietic stem cell transplantation (HSCT) increased neutrophil and lymphocyte numbers, enhanced T-cell regeneration, and increased disease-free survival without exacerbating graft-versus-host disease [54]. Clearly a peripheral effect of GnRHa on the immune system may influence the effect of chemotherapy both on the primary goal of cancer treatment, but also on any putative fertility preservation outcomes.

## Hypothetical mechanisms of ovarian protection

Blumenfeld hypothesized additional potential mechanisms for GnRHa to protect ovarian function: via decrease in secretion of growth factors by suppression of FSH-dependent follicular turnover; potential upregulation of sphingosine-1-phosphate, an antagonist of the proapoptotic second messenger ceramide; or by protection of undifferentiated germ-line stem cells should they be proven to exist in humans [2]. Whether these putative mechanisms require further study will be predicated upon whether GnRHa are proven to protect not only the resumption of menses, but also the retention of true ovarian function with both sustained cyclical steroidogenesis and live birth in those women desiring future parenthood.

Given the many and varied influences on different organ systems attributed to GnRH both centrally and peripherally, robust evaluation of a role for GnRHa in fertility preservation during chemotherapy awaits adequately powered randomized controlled trials powered for the true outcomes of interest: live birth, time to menopause and survival. The data amassed so far supports further investigation, but the lack of randomized data on the effect of GnRHa on long-term ovarian function and survival outcomes leaves concern regarding potential harm.

While the role of GnRHa in fertility preservation during chemotherapy is being clarified in randomized controlled trials, women facing gonadotoxic treatment should also receive counsel regarding the more mature fertility preservation options already proven to result in live birth. These include the well-established embryo cryopreservation, as well as the newer and still investigational oocyte and ovarian tissue cryopreservation.

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Chapter 19

# Ovarian transposition

Carrie A. Smith, Erin Rohde and Giuseppe Del Priore

The relocation of ovaries for their protection in women diagnosed with cancer in the pelvis was mentioned as early as 1958 by McCall *et al.* [1, 2]. At that time, the procedure was termed oophoropexy and considered to be revolutionary, controversial and "cutting edge" fertility preservation. Now, over 50 years later, this procedure has another, perhaps more accurate name, of ovarian transposition. Paradoxically, it is still considered controversial as the next generation of physicians becomes experienced in its benefits and limitations.

Although the discussion of ovarian transposition has not changed much in the last 50 years, women's reproductive behavior has, making transposition a more important topic. This is because women are, on average waiting longer to have their first child, desiring subsequent children at later ages, and are becoming more aware of their available fertility options [3–5]. Although cancers of the pelvic region still remain rare in women of reproductive age, their incidence increases with age, thus leaving women with delayed first pregnancy more vulnerable to the possible loss of fertility.

The increase in the number of potentially affected women can be estimated based on cancer incidence and fecundity. For instance, even if the general incidence of primary pelvic malignancies is approximately 1/10 000, then a delay from age 24 to age 25 in the median age of first conception may expose a significant number of women to a cancer diagnosis before first planned or desired pregnancy. Conservatively, there are at least 10 million reproductive age women in the USA with a fecundity rate of 20% or more. Using different underlying assumptions will affect the number in a predictable manner. Ranges of high and low estimates can thus be assumed for planning and policy issues. Regardless, even the most optimistic assumptions will result in a predicted yearly increase in the number of women at risk, all potential candidates for fertility preservation and, if indicated, ovarian transposition (Table 19.1).

**Table 19.1** Suggested eligibility criteria for ovarian transposition

- Age < 40 years
- Cervical cancer < 3 cm in diameter
- Cervical cancer confined to the cervix
- No evidence of lymph-vascular space invasion
- No evidence of lower uterine segment involvement
- Early stage (IA) well-differentiated (grade 1) endometrial cancer
- Any malignancy that requires pelvic radiation therapy with or without hysterectomy

In addition to the change in women's reproductive behavior, their reproductive expectations have changed as well. Shover *et al.* found that 75% of women with cancer expressed a desire to have children in the future, a majority of whom continued to desire children even if they were to die young [5]. This statistic has been reproduced and reported by multiple investigators in a variety of disease states. Although estimates vary considerably mostly due to study design and questionnaire nuances, the results can never be interpreted as only affecting an insignificant minority. More often, any conclusion that minimizes the importance of fertility preservation, and therefore a therapeutic intervention such as ovarian transposition, actually reflects an investigator bias [6].

This is demonstrated clearly in a series of three papers reporting potentially affected cervical cancer patients over a span of 20 years in the literature. All three investigator groups defined the potential number of patients interested in fertility preservation based solely on age. In the first paper, reported in 1990 by Maddux *et al.*, the fertility interested group was defined strictly by age <25 [7]. Using this cut off, only

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2% of cancer patients would be potential ovarian transposition candidates. A few years later, Mariani *et al.* used age <35 which increased the potential number of affected patients to 10% [8]. Finally, a New York city group reported in 2004 that more than 40% of their patients would be interested in fertility preservation [9]! This group used age 40 as the cut off, reflecting a significant change in definition and perspective from the earliest study. Obviously none of these studies had an objective basis for their methods. Instead the arbitrary cut offs were based on contemporary and fluid definitions of reproductive age. Because of the uncertain estimates of the size and definition of the affected population, all patients should be counseled regarding fertility preservation, including the use of ovarian transposition.

Ovarian function is compromised when damaged during surgery, exposed to radiation, and/or chemotherapy. Studies have shown that the amount of radiation to eliminate 50% of a young woman's primordial follicles is 2–4 Gy, with 20 Gy of radiation exposure resulting in complete and permanent ovarian failure [10–14]. Pelvic radiation to treat gynecological cancers can be as much as 70 Gy planned prescribed dose to the ovarian fossa. Given the lack of precision inherent in radiation planning versus delivery, the actual received dose that the ovaries absorb can be significantly higher.

In addition, after decades of a relatively stable delivery system using linear accelerators, radiation delivery has developed many more options designed to improve cancer care, increase cures and decrease toxicity [10–12, 14]. However there is little to no information on the affect of these modalities on *in situ* or transposed ovaries. The general impression and hope is that there will be less toxicity; however, caution must be used in counseling patients treated with these latest modalities that transposition is or is not indicated until data become available.

Chemotherapy has been found to have a highly variable chance of acute ovarian failure [14, 15]. There is also a highly variable increased long-term chance of premature ovarian failure [16]. As discussed below, the variation in reported chemotherapy affects is due largely to study design and outcomes. Ovarian transposition done to preserve ovarian function may paradoxically increase the susceptibility to subsequent toxic exposure. This is only a theoretical concern based on the reports of ovarian failure following other pelvic surgeries, especially hysterectomies. Disruption of normal ovarian vasculature through hysterectomy is associated with changes in serum estradiol and follicle stimulating hormone (FSH) levels in retained ovaries. It is possible that ovarian transposition could result in a thrombosed or otherwise compromised ovarian vascular blood supply. This could, again theoretically, render the ovaries more vulnerable to the toxic effects of chemotherapy.

It is important to note the limitation on assessing ovarian function and reserve when considering ovarian transposition. There is no universally accepted metric to determine current ovarian function or future potential. Immediate assessments including estradiol, follicle counts, FSH and similar measures correlate poorly with reproductive, i.e. fertility, potential. Often, resumption of menses is used as a very crude assessment of ovarian function. In addition to the poor correlation between menstruation and fertility potential, this data is often reported at arbitrary follow-up intervals such as 6 months. When counseling a patient regarding transposition, the limitation of the data should be addressed to avoid unrealistic expectations.

Once the decision to perform ovarian transposition has been reached, the surgical method must be considered [17]. There have been several proposed methods, including open laparotomy or minimally invasive. If technical reasons such as body size and shape allow, minimally invasive approaches are preferred. Each is relatively simple with documented pregnancies after the transposition.

Another alternative often not considered today is a whole ovary, vascular anastomosis to an extraperitoneal site [18]. The technique is often referred to as a "transplant" or a "heterotopic autotransplantation" procedure because it shares characteristics of other common organ transplantation such as the kidney transplants. In fact, it can be thought of as a "transposition" with important differences. The addition of a vascular anastomosis is not a significant difference. The anastomosis is an easy routine procedure for transplant, vascular and plastic surgeons. What is significantly different is the microenvironment of the alternative locations. It is possible that the relative zero gravity of the abdominal peritoneal cavity is an essential, or at least an important, parameter in normal ovarian function.

Alternative transposition locations including the upper extremity [18] have not been shown to be successful by resulting in pregnancy, but have shown

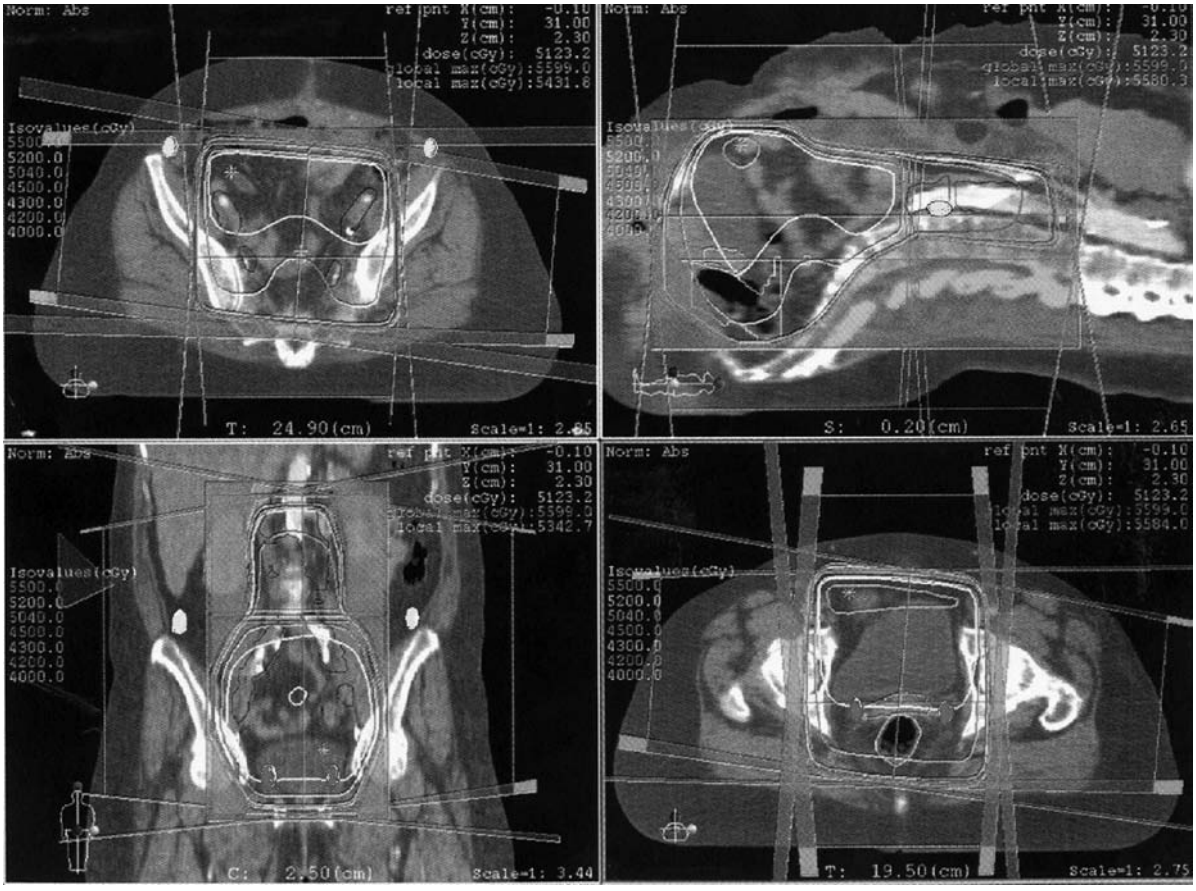


**Figure 19.1** Radiation field. See plate section for color version.

return of ovarian function, including growth of follicles. Limited function and utility have been reported only as isolated cases. Alternative sites may be considered as part of a research protocol. Ovarian transposition to the upper abdominal cavity using superior epigastric vessels or internal mammary should be investigated carefully.

Currently the relocation of the ovaries with an intact and uninterrupted gonadal vascular bundle to another intraperitoneal location is the only practical option [12, 13, 17]. This can be accomplished using minimally invasive (robotic, single site or conventional laparoscopy). The goal is the same, to remove the ovaries and typically fallopian tube, to outside the pelvis (Figure 19.1). Typical radiation borders include the ovarian fossa and extend beyond the true pelvis. For this reason, there is no absolutely perfect location for every disease and radiation treatment plan. Surgeons should consult with the requesting radiation therapist to understand the target tissue and dose. Together, surgeon and radiotherapist can select the best location [19].

In general, for gynecological malignancies, cervical and uterine cancers are the most likely indications for adjuvant or definitive radiation treatment to the pelvis, but pelvic radiation is also done for Hodgkin's lymphoma, pediatric sarcomas and rectal cancer [11]. Vulva cancer has limited indications for whole pelvic radiation and disease is relatively rare in reproductive age women. Ovary cancer is very unlikely to require radiation therapy while still being an indication for transposition. For a unilateral, well-differentiated, radiation-sensitive dysgerminoma, it is theoretically possible to consider transposition of the contralateral ovary but highly improbable.

For the typical cervical and endometrial cancer diagnoses, the treatment fields follow closely the lymphatic drainage of the primary tumor. For instance,

since obturator lymph nodes are often involved with, or at risk for, metastatic cancer, the radiation field will include the obturator fossa and extend laterally approximately 2 cm (Figure 19.1). The target area then moves more medially and cephalad. This superior field area allows the para-colic gutter to be a relative sanctuary for the ovary [19–21]. Because of the relatively common use of pelvic radiation after endometrial or cervical cancer, ovarian transposition may be considered for all these patients at the time of the definitive cancer operation.

The ascending and descending colon make up the medial border of the acceptable location for the transposed ovary. The inferior border is defined by the anterior iliac crest. There is no lateral or cephaled limit to where the ovaries can be relocated to. This direction is only limited by the moblization of the gonadal vessels, i.e. the infundibulopelvic ligament [22]. There may be an advantage to moving the ovaries as far as possible from the target tissue to minimize scatter. Moving the ovaries as far as possible may also be an advantage in preventing the migration of the transposed ovary back into the field.

Possibly due to the effect of gravity and lax unsubstantial points of fixation, a significant number of ovaries are found to be back in the radiation field on postoperative imaging [19–21]. Fixation on the abdominal wall requires incorporation of more than just the peritoneum. The underlying fascia and muscle should be part of the fixation when using suture or any other method. The ovarian bundle is more limited in its choices for fixation. Too aggressive a purchase may occlude vascular supple. Too flimsy a purchase may slip and become loose.

It is important to attach radio-opaque staples to the ovaries or perform an MRI to detect this potential movement [23, 24]. There is no question that rigorous multiple sutures may be more likely to retain the ovaries in the intended location. However, obviously, the more substantial the sutures the greater the risk for ovarian ischemia from occluded or kinked blood vessels. This is a similar concern regarding the tension used to move the ovary ever further laterally or cephalad. Tension on the vascular pedicle may lead to compromise of ovarian function [20, 21, 25].

It is hard to estimate a true incidence of ovarian failure after transposition because of the small numbers in the case series reported [20, 26, 27]. Other limitations include the variable case ascertainment used. Too often "menses at 6 months" or some other arbitrary definition of success is used. However, the consensus estimate is that a substantial minority of transposed ovaries will cease to function after transposition. Techniques have been devised to minimize this complication.

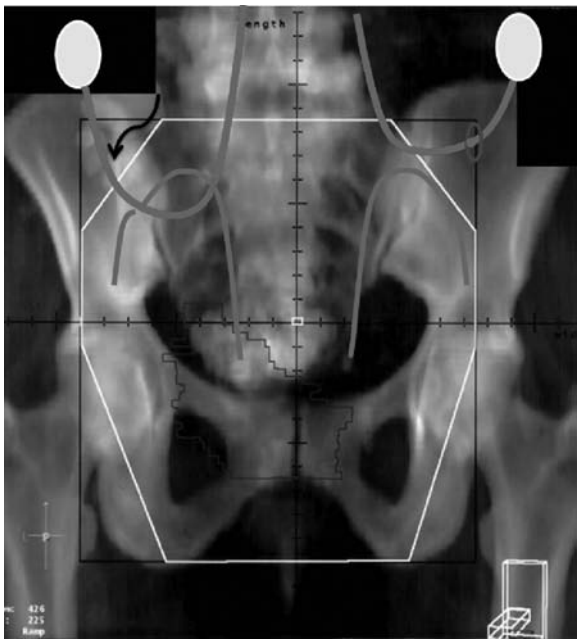

**Figure 19.2** Surgical anatomy. See plate section for color version.

For instance, a report indicated that nearly all ovarian function can be preserved, at least in the short term, using a retro-peritoneal approach. Briefly in this technique, the ovary and its distal blood supply are brought retroperitoneal superior and lateral to the distal incised utero-ovarian ligament. A window is made in the paracolic gutter peritoneum and the ovary gently pulled through this opening (Figure 19.2).

This technique theoretically improves outcomes by preventing ovarian peritoneal inclusion cyst as the ovary retains its normal relationship with the peritoneal surface [21, 28]. In addition, this technique avoids the creation of a potential internal hernia by a taunt gonadal pedicle. When the ovary is directly attached to the abdominal wall, it should be done so with as little tension as possible on the gonadal vascular bundle. Ideally it should lie flat against the side wall without tension. There have been no direct comparisons of the two techniques.

How the ovary is attached to the lateral abdominal wall varies by operator preference [22, 24, 25]. Suture has an advantage of being able to precisely place

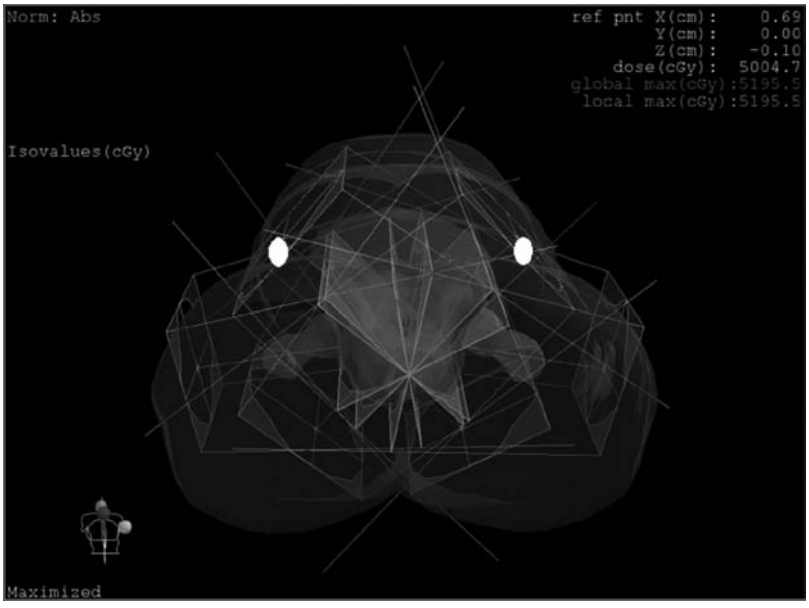


**Figure 19.3** Intensity modulated radiation therapy (IMRT). See plate section for color version.

the purchase on both the ovary and the abdominal wall. However, because of the relative difficulty of tying and suturing laparoscopically, alternatives have also been reported. These alternatives include hemostatic clips and hernia-type staplers. The hernia staplers are very secure and purchase the underlying fascia easily. The hemostatic clips require a secondary incision be made to allow it to be "slipped" under the fascia. Any of the above mentioned techniques can be performed using either open or minimally invasive techniques.

No location is entirely safe from the internal scatter radiation dose that occurs during all conventional treatments. There is a theoretical reduction in this scatter dose using enhanced delivery modalities such as intensity modulation (Figure 19.3). However, there have been no reliable reports on ovarian function after these advances in radiation therapy.

There have been reports of complications after transposition [28]. These include the usual postoperative issues of infection, bleeding and hernias. Especially disappointing complications include ovarian failure supposedly due to ovarian vein thrombosis and torsion. Reoperation for peritoneal or ovarian cyst is more common than in the general population.

There have also been reports of spontaneous conception after transposition including the intact Fallopian tube [28, 29]. In certain patients, the Fallopian tube can be stretched sufficiently to allow the connection to the uterus to be retained. In most cases, a tension-free transposition requires transaction of the utero-ovarian ligament and transaction of the medial Fallopian tube.

In some of the cases of spontaneous conception, the ovaries and tubes have been documented to have returned to their original natural position after completion of the planned radiation therapy. However, pregnancies have been reported while still in the transposed location. There are even reports of planned temporary relocation of the ovaries in hopes of promoting spontaneous conception [23, 29]. It must be remembered that most women is whom transposition is indicated, either the uterus is removed or it is radiated often destroying the endometrium. The reports of spontaneous pregnancy usually involve patients with medial transposition behind the uterus, with central shielding and lateral radiation therapy.

Ovarian function is almost guaranteed to be entirely lost without some intervention before pelvic radiation therapy. Ovarian transposition is a relatively simple option that should be considered with all patients at risk for ovarian failure due to radiation. Unfortunately it is not possible to guarantee preservation of ovarian function or future fertility using any technique [30]. Alternative continue to be developed including ovary cryopreservation and other cutting-edge options [31–34]. Until then, transposition is a reasonable option.

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Chapter 20

# Fertility-saving surgery for cervical cancer

P. Mathevet and A. Ciobanu

## Introduction

Patients with early cervical cancer (FIGO stage IB1 or less) are conventionally considered treated with a surgical approach while those with more advanced disease are treated by radiotherapy with concurrent chemotherapy. Surgical treatment usually involves a radical hysterectomy with pelvic lymphadenectomy except for those with early microscopic disease (FIGO stage IA1) where conisation or extrafascial hysterectomy ± lymph-node dissection would be sufficient. With the good implementation of cervical screening programs in most developed countries, more women are presenting with early cervical invasive disease. For these women, the long-term prognosis is good, with survival of over 90%. With good long-term survival, preservation of fertility potential and quality of life become more important issues and have been the focus of recent surgical advances in the treatment of cervical cancer.

For FIGO stage IA1 without lymph-vascular space involvement, cold-knife conisation with a complete lesion resection is recommended as a fertility-preserving treatment, since the rate of parametrium and lymph-node involvement is negligible. For larger tumors, or in case of lymph-vascular space involvement, due to risk of lymphatic extension, resection of the pelvic lymph nodes and of the proximal part of the parametrium is recommended. For these patients, preserving the fertility was a challenging option until recently.

The recent development in surgical oncological technologies aims at improving their effectiveness while decreasing their morbidity. Therefore, the endoscopic and in particular laparoscopic techniques were introduced in surgical oncology. With these concepts of optimization of the treatments and reduction of their consequences, the gynecologists also proposed fertility-sparing techniques for young women with cancer and a desire to preserve reproductive function. Twenty years ago it was completely impossible to have a child after treatment for cervical cancer from an oncological and surgical point of view. This context emphasizes the originality of the step of Professor Dargent, when he created and carried out the first surgical operation aiming at preserving the fertility among young women presenting with early cancer of the uterine cervix.

While the first intervention was carried out at the end of the year 1986, the first publications were made in 1994 [1], allowing us to verify the absence of oncological risk associated with this "modern" surgical operation. Thus, in the 1990s, Dargent's operation began to be distributed worldwide, with the help of British, Canadian and American surgical teams in particular. These different teams [2–7] have confirmed our experience of the radical trachelectomy and and explored the opportunities to achieve a pregnancy and also the risk of relapse after this type of operation.

Currently, more than 500 women have benefited from this intervention with relatively reduced morbidity worldwide and a perfectly controlled risk of relapse. More importantly, they have had the possibility of achieving a pregnancy, and having healthy children, following conservative radical treatment of early cervical cancer.

Dargent's operation is the realization of a laparoscopic pelvic lymph-node dissection associated with a radical cervical amputation through a vaginal approach [1, 8]. This radical resection includes a total amputation of the uterine cervix associated with the vaginal cuff along with the proximal part of the parametrium. Concerning the uterine cervix, the

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
Published by Cambridge University Press. 

resection is comparable to that which can be obtained from a Wertheim's operation type Piver II. However, the concept of radical cervical amputation is not a totally new concept since some authors had already proposed it, for instance, by Aburel and Nowak in the 1940s and 1950s. But no one achieved pregnancy after the completion of this type of intervention that had been carried out through an abdominal approach. It was therefore the development of laparoscopy (which limits adhesions and reduces the risk of infertility associated to the radical trachelectomy) which helped obtain pregnancies and live births following surgical treatment for early cervical cancer.

## Operative technique

In our team, the operation always begins with a laparoscopic pelvic lymph-node dissection, which includes sentinel node identification. This technique of the sentinel node identification discovers, in a significant proportion of cases (15–20%), lymphatic drainage channels with unusual locations of sentinel nodes. Identification of the sentinel node allows the realization of frozen sections, their result being of paramount importance for the next surgical step. Indeed, if the frozen-sections evaluation is positive, the planned radical trachelectomy is cancelled and the patient receives a full laparoscopic pelvic, common iliac and paraaortic lymph-node dissection followed by radiotherapy and chemotherapy. Detection of the sentinel node is performed with a dual technique of isotopic colloid particle (Nanocys®) and color (Patent Blue®) injection in the cervix (in the four quadrants) (Figure 20.1), and then the sentinel nodes are identified through laparoscopy (Figure 20.2). This identification is followed by a full pelvic lymph-node dissection performed from the obturator membrane until the iliac bifurcation, with the removal of all external iliac lymph nodes (below the vein and at the contact of the external iliac vein and artery). In the case of a tumor larger than 2 cm in maximal diameter, the cellulo-adipose tissue of the distal parametrium is dissected through laparoscopy (corresponding to a lymph-node dissection of the parametrium).

The next step is that of the radical trachelectomy performed through the vagina [8]. The steps of this operation reproduce those of the intervention of Schauta–Stoeckel. Dargent's operation includes the following steps. The first step is the realization of a vaginal cuff. It is rarely necessary to remove more than

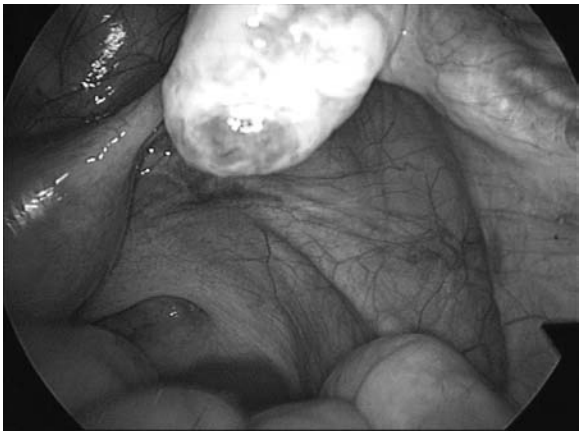

**Figure 20.1** Visualization of right pelvic lymphatic channels through laparoscopy after Patent Blue® injection in the cervix. See plate section for color version.

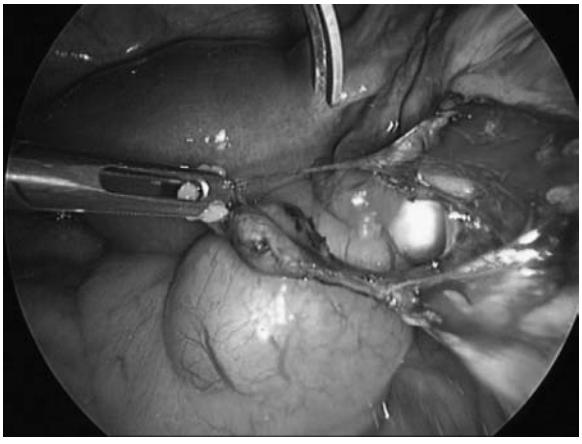

**Figure 20.2** Dissection of a right pelvic sentinel node. See plate section for color version.

1–2 cm of vaginal mucosa. Thus, a rim of vaginal tissue is delineated circumferentially using 6–8 Kocher forceps (Figure 20.3). In order to reduce bleeding and to help for the following dissections, 20 cc of xylocaine plus epinephrine is injected at regular intervals in the vaginal fold. A round incision of the vaginal wall is made just above the Kocher forceps. Once the incision is fully made, the Kocher forceps are removed and the anterior and posterior edges of the vaginal cuff are grasped together with 4–6 Chroback clamps in order to isolate the cervix and to prevent tumor spillage. These clamps allow good traction onto the operative specimen in order to help the following steps. Then dissection of the vesico-vaginal septum is performed

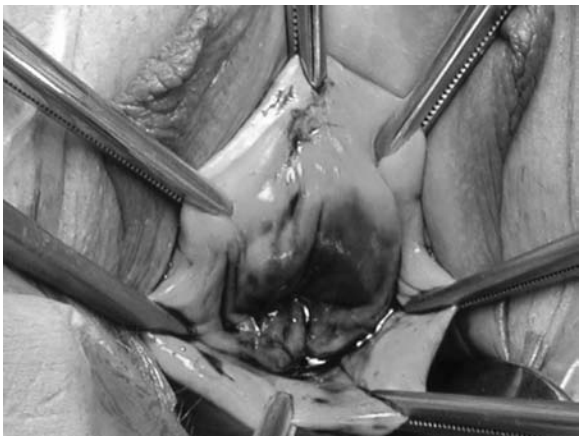

**Figure 20.3** Performance of the vaginal cuff. See plate section for color version.

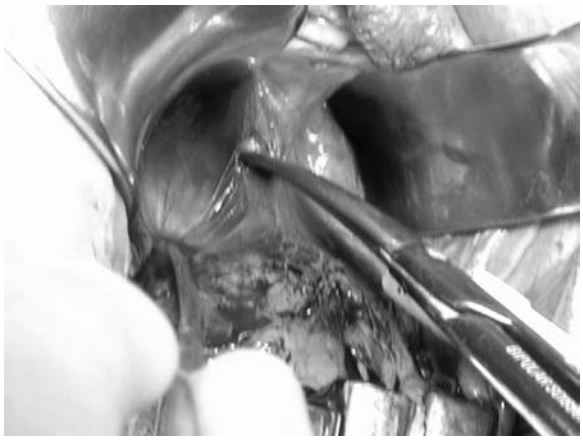

**Figure 20.4** Dissection of bladder pillars and identification of the left ureter. See plate section for color version.

on the midline with scissors. The space is usually avascular and, when fully dissected, the bladder base is mobilized with a vaginal retractor. The following step is the opening of the para-vesical space. On the left side, the opening is obtained while dissecting along the vaginal wall at 3 o'clock level. It should be noticed that the upper part of this space has been dissected through laparoscopy helping the vaginal approach. A retractor is put into the para-vesical space, and palpation of the bladder pillar and the ureter is performed between the surgeon finger and the retractor. The precise location of the ureter can easily be defined. And so, the bladder pillars are excised after coagulation. The goal of this dissection is to evidence the ureter (Figure 20.4), and then to fully cut the bladder pillars in order to free the bladder base and to move away the lower part of the ureter from the operative field. The same procedure is performed on the patient's right side. The following steps are then performed on the posterior aspect of the operative specimen. It first consists of opening the Douglas pouch. This opening is performed with scissors on the midline, as for a simple vaginal hysterectomy. Then a vaginal retractor is put in place in order to evidence the utero-sacral ligaments. These ligaments are coagulated and divided leading to opening of the para-rectal space and identification of the posterior aspect of the parametrium. Then the next steps are modifications of the ones performed during the Schauta operation. Indeed, the division of the parametrium is carried out while preserving the uterine artery. With retractors in place in the para-vesical and para-rectal spaces, the anterior and posterior aspects of the parametrium and the ureter are evidenced. Then the parametrium is divided while preserving the arch of the uterine artery. The division of the parametrium can be made by clamping and ligation or with the use of electrosurgery devices. The ureter should be fully dissected and left under the view while performing the parametrium division. This procedure is performed on both sides. The following step is the ligation of the cervico-vaginal vessels with preservation of the uterine artery (Figure 20.5). Then the uterine cervix is cut with a cold knife at the level of the lower part of the isthmus (Figures 20.6 and 20.7). The operative specimen includes the entire cervix until the isthmus, the proximal part of the parametrium about 2 cm in length and the vaginal cuff (Figure 20.8). Frozen sections are performed onto the upper margin of the operative specimen in order to be sure that the section is in healthy zone. If this margin is not clear, the radical trachelectomy is transformed into a Schauta operation. Concerning the operative specimen, a final pathological analysis will be performed following the same procedure as the one recommended for cone specimens.

The Douglas pouch is then closed with a purse-string absorbable suture. In the case of future pregnancy, a permanent cerclage is set around the uterine isthmus. A non-absorbable polyethylene tape is usually used. The cerclage is made following the Benson technique with the knot lying posteriorly (Figure 20.9). Then the vaginal anastomosis is carried out by two Sturmdorff sutures and two angle sutures with absorbable sutures.

In the postoperative period, a bladder catheter is left in place for 4 days. On the 4th postoperative

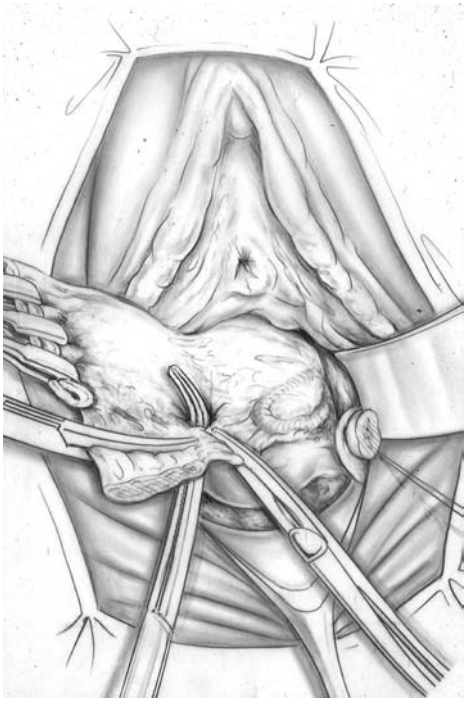

**Figure 20.5** Drawing of the ligation of cervico-vaginal vessels.

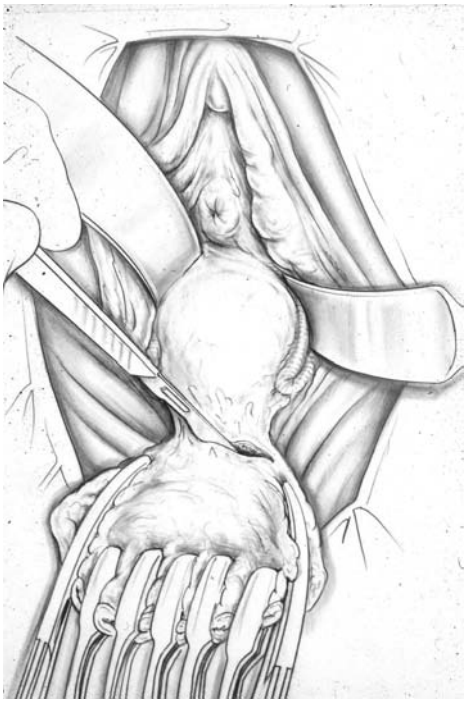

**Figure 20.7** Drawing of the section of the operative specimen at the level of the uterine isthmus.

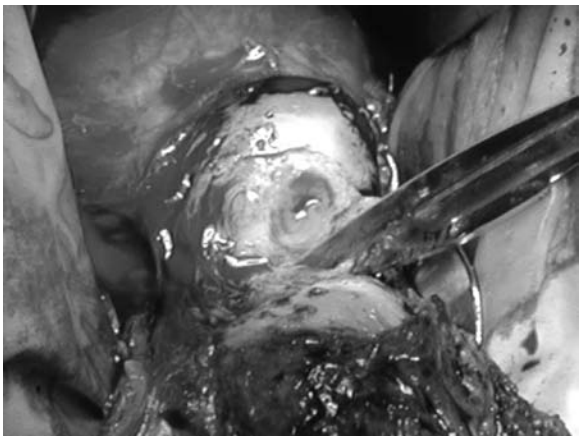

**Figure 20.6** Section of the operative specimen at the level of the uterine isthmus. See plate section for color version.

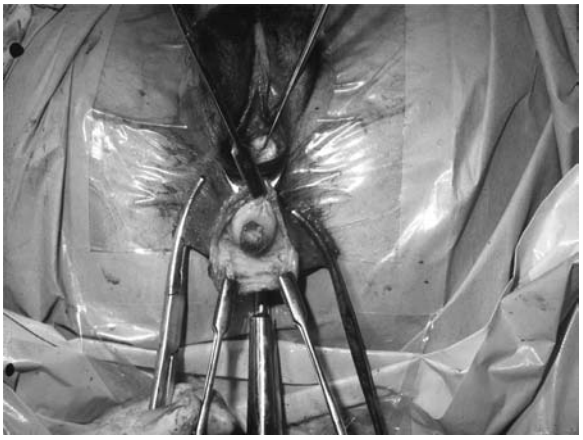

**Figure 20.8** Operative specimen of a radical trachelectomy showing the vaginal cuff and the proximal parametrial resection. See plate section for color version.

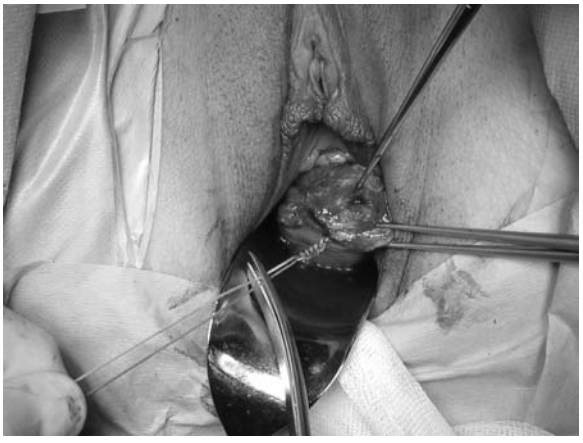

**Figure 20.9** Set up of the isthmic cerclage. See plate section for color version.

day, the ablation of the catheter is performed after checking the bladder function, allowing the patient's discharge.

Usually, we advise patients to wait 2 years before considering a pregnancy (as the majority of relapses occur within 2 years). However, in the event of lesion of good prognosis and/or patients approaching the limit of childbearing age, this time can be shortened to 6 months.

## Our experience

In our department, from December 1986 to December 2008, Dargent's operation was proposed to 171 patients. For 11 of these patients, the final intervention was changed either because of a lymph-node invasion (6 cases) or because of isthmic involvement by the tumor (5 cases). Thus, 160 radical trachelectomies were carried out. The average age of the patients was 31.5 years. The proportion of nuliparous patients was 56%.

The initial stages of the tumors were: stage IA1 or (+ lymph vascular space invasion) IA2 in 38 cases (24%); and stage IB1 in 122 patients (76%). The histological types were: squamous in 123 cases (77%), glandular in 35 cases (22%) and other types (of which 1 was neuroendocrine) in 2 cases (1%). The maximum tumoral size was $<2$ cm in 130 cases (81%) and $\geq 2$ cm in 30 cases (19%). The presence of lymph vascular space invasion (LVSI) was found in 51 cases (32%).

The mean operating time was 192 min (including the associated laparoscopic pelvic lymph-node dissection). The only intraoperative complications observed (2 cases, 1%) were a ureteral laceration and a bladder breach, which were sutured without further damage. The average hospital stay was 4.6 days. During the postoperative course a notable complication was observed in 14 patients (19%). Nine (6%) patients presented a postoperative bleeding requiring a surgical revision. Two pelvic lymphocysts required a surgical marsupialization. Two abscesses (pelvic and parietal) were drained. A case of uretero-vaginal fistula required a ureteral anastomosis without sequellas. It should be noted that the rate of complication was significant, especially in the first-operated cases because of the experimental character of the surgical technique. Concerning the last 70 patients who went through surgery, no operative complication was observed and the postoperative course was marked by 3 (2%) of the 14 complications previously described.

In nine patients, adjuvant radiotherapy was recommended either because of a lymph-nodal or parametrial tumor involvement at the final histological examination (seven cases) or because of a massive LVSI (two cases). The average follow-up of the patients is 92 months. To this date, 8 (5%) relapses were observed, leading to death in 6 cases. A neuroendocrine case of cervical cancer presented 2.5 years after surgical treatment with distant metastases leading to the death of the patient. Four nodal relapses were observed (located in the latero-pelvic, common iliac and para-aortic areas). Despite the treatment of these recurrences (surgery and radiotherapy), three of these patients died. A patient with a stage IB1 adenocarcinoma presented a central-pelvic relapse on the uterine isthmus. She was operated on and irradiated and is currently doing well. Two patients presented relapses in the parametrium and unfortunately could not be saved despite loco-regional treatments.

The prognostic factors of the relapses were evaluated. After exclusion of the case of neuroendocrine cancer, which represents an inadequate indication of radical trachelectomy, the only statistically significant factor for relapse is tumoral size. Thus, in the event of maximum tumoral size $>2$ cm, 6 relapses (20%) were observed against 1 relapse ($<1\%$) when the size was $<2$ cm ($P < 0.05$). The presence of LVSI is associated with an increased risk of recurrence, but the difference is not statistically significant: 5 relapses (10%) in the event of the presence of LVSI, against 2 relapses (3%) in the absence of LVSI ($P =$ NS). The histological type has no effect on the risk of recurrence.

The last update of the pregnancies obtained after radical trachelectomy was carried out in 2005. At that time, we had observed the birth of 49 live healthy children following radical trachelectomy performed in our department. All the childbirths were carried out by cesarian section. The evaluation of the fertility after Dargent's operation made it possible to find infertility in relation to the operation (primarily by isthmic stenosis) in 16% of the cases. The rate of late miscarriage is relatively important in our practice since it is 15% of all the pregnancies. However, this rate of late miscarriage was lowered by the introduction of various procedures: the use of isthmic cerclage, which made it possible to halve the rate of miscarriage, and then the closing of the uterine cervix according to the Saling procedure, which made it possible to reduce the rate of late miscarriage in our experience from 21 to 12%.

So, the results of our experience demonstrate that the radical trachelectomy is a valid response in the treatment of young women affected by an early cervical cancer and wishing to preserve their fertility. Dargent's operation does not appear to increase the risk of cancer relapse and allows, in a significant proportion of cases, pregnancies and, especially, live births. Our experience has been also confirmed by data from other teams in the world.

## Experience worldwide [2–7, 9, 10]

Radical trachelectomy has been performed by numerous surgical teams across the world, and currently more than 500 patients have benefited from this operation. Recently, a survey of the experience of most teams practicing this intervention throughout the world was presented. Thus, in total, 557 patients (mean age = 31.5 ± 4.4 years) have benefited from radical trachelectomy. The stages of the cervical cancers were: stage IA in 34% and stage IB1 in 76% of patients. The observed recurrence rate was 28 (5%) of cases. In 3 cases the recurrence was only cervical intraepithelial neoplasia (CIN) or vaginal intraepithelial neoplasia (VaIN) and therefore 25 (4.5%) women had an invasive recurrence. Twelve (2.1%) women died from this recurrence and 4 others are living with progressive disease.

Concerning pregnancies after radical trachelectomy, the data are based on 445 patients for which the information was available. The late miscarriage rate is 15% (22 cases identified) and 125 live healthy children were born by cesarian section. Thus, the results of other teams confirm the validity of the technique in terms of oncological risk, possibility of pregnancies and live births after completion of a radical trachelectomy.

## Discussion

Dargent's operation or radical trachelectomy was invented to enable preservation of fertility among women with early cervical cancer. The current follow-up of the first cases over 20 years confirms the hopes of Professor Dargent: women benefiting from this operation have an excellent opportunity to have a healthy living child after this treatment without increased oncological risk [8]. The benefits of Dargent's operation are linked to the laparoscopic approach, which reduces the risk of adhesions on pelvis organs and the vaginal route that allows the preservation of uterine body and its optimal vascularization [6]. The limits of this procedure are mainly related to the vaginal approach, which may be difficult for some surgeons not trained in this approach.

Also, following the excellent results achieved by the Dargent's operation, several teams have proposed operating variants through laparotomy [11–16] or laparoscopy [17, 18]. These modifications are subject to criticism because they are usually associated with the section of the uterine arteries and, thus, with a partial devascularization of the uterus. In addition, the laparotomy approach is associated with an increased risk of adhesions. Data from the literature also concur that the rate of pregnancy after radical trachelectomy by laparotomy or laparoscopy is lower than by the vaginal approach [19]. Currently, we believe that only the original operation described by Daniel Dargent must be promoted in order to preserve the fertility in young women affected by an early cervical cancer.

The experience of pioneer teams around the world helps to refine indications of this operation. Preferential indications are represented by cervical cancer stage IA2 and IB1 of $< 2$ cm maximal diameter. Unusual histological types must be excluded from this treatment. Moreover, lesions extending high in the endocervix are a contraindication to this operation, which may be too close to the upper limit of the tumor when performing the isthmic section. It is therefore necessary that the patient planned for a radical trachelectomy must have a preoperative exploration with colposcopy and pelvic MRI to exclude significant extension of the lesions in the parametrium or the endocervix [8]. In particular, MRI should define the extent of the lesion in relation with the isthmus, considering that at least 8–10 mm of normal cervix above the lesion should be obtained at surgery.

Currently, we have enough data to say that Dargent's operation is a valid surgical alternative from an oncological and obstetrical point of view, but many questions remain unanswered:

1. *Should a closing hysterectomy be performed after the patient has obtained the number of children she wanted?* Only the risk of centro-pelvic recurrence in long-term studies allows us to answer this question. Our experience enables us to estimate that this risk is very low, even negligible. In

addition, the realization of a hysterectomy after radical cervical amputation is not an easy operation. We believe today that there is no need to consider a closing hysterectomy among these patients. However, this data should be modulated according to the histological type of the initial lesion. Indeed, it is known that cervical adenocarcinomas sometimes have a multifocal character which could potentially foster a possible isthmic recurrence in these patients.

2. *What are the best treatment options in order to increase the patient's chances of obtaining a term pregnancy after a radical trachelectomy?* Indeed, after a Dargent's operation the rate of late miscarriage as well as premature birth is significant. Two main mechanisms are involved in the increased risk of those obstetrical complications: mechanical incompetence of the cervix and subclinical membrane infection linked to the significant reduction in the amount of cervical mucus. Isthmic competence can be improved by making a definitive isthmic cerclage performed at the same time as the trachelectomy. Concerning the infectious factor, we propose closure of the cervix following the Saling technique during pregnancy. This surgical procedure allows us to isolate the membranes of the vaginal bacteria and reduces the risk of membrane contamination and chorio-amniotitis. Some authors also advocate an antibiotic treatment throughout the pregnancy [20]. However, the evaluation of the benefits of these different therapeutic approaches is practically difficult. Besides, prophylactic approach by monitoring the residual "cervical" length by ultrasound has not yet received appropriate studies. Also, time off work and bed rest may be important as the uterus gets heavier.
3. *Is the radical trachelectomy too large for small cervical cancers < 2 cm in diameter? I.e. can we achieve a less radical treatment in the case of very early cervical cancer: simple amputation of the uterine cervix or even a large conization?* It's a recurring question in the support of early cervical cancers. But data from the literature are very poor in this area. Attempts to reduce surgical morbidity in the 1970s by Professor Burghardt failed due to an important and dangerous recurrence rate. However, radiological exams and histological evaluation techniques have progressed and we currently better understand in which cases you can reduce surgical aggressiveness: invasive tumor of < 2 cm in diameter without the presence of LVSI. Recent works have been presented in this area by a Czech team [21, 22] and experiments have been started in the USA. However, to demonstrate equivalence in terms of oncological risk with less extensive surgery compared to radical surgery, statistically the numbers of patients must be very important and therefore make this type of study hardly feasible.
4. *Can we consider the realization of conservative treatment for stage IB tumors larger than 2 cm?* At present, this indication is not accepted by most teams that practice the radical trachelectomy. However, initially, a significant proportion of patients were performed on for tumors of this size, either because of the intense desire of the patient or because of inadequate performance of the MRI. More accurate analysis of women with tumors between 2 and 4 cm and treated with radical trachelectomy shows a substantial recurrence rate (to the order of 15%) but comparable to that achieved with the performance of Wertheim's operation in a similar group of patients with cervical cancer. So we do not create a major oncological risk by performing the radical trachelectomy in this particular group of patients having a tumor 2–4 cm in diameter. However, several teams are working on experiments of neoadjuvant treatments in order to reduce tumor size, and therefore to be able to perform conservative surgery in patients with stage IB cervical cancer larger than 2 cm. These preliminary experiments using neoadjuvant chemotherapy (usually a polychemotherapy with Taxotere®, Holoxan® and Cisplatine®) lead to excellent response rates and a reduced risk of recurrence (Figures 20.10 and 20.11) [23]. Moreover, these patients have a good recovery of ovarian function and their fertility seems preserved, with already isolated cases of pregnancy described [24, 25]. This approach is interesting and probably will lead to therapeutic developments in the future.

## Conclusion

Dargent's operation was developed during a period of reduction in aggressive operative techniques in the

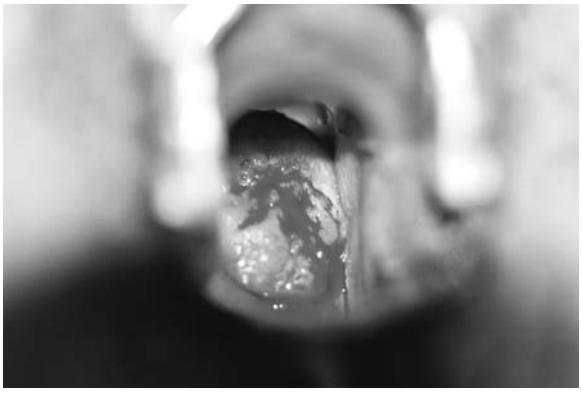

**Figure 20.10** Pre-treatment epidermoid cervical carcinoma stage IB2 (45 mm) in a 25-year-old young woman, neoadjuvant chemotherapy is planned. See plate section for color version.

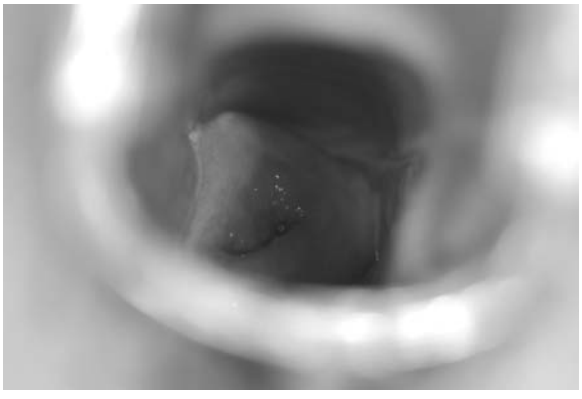

**Figure 20.11** Same patient after four courses of neoadjuvant chemotherapy: complete regression of the lesion. See plate section for color version.

field of gynecological cancers. This operation allows the preservation of the fertility of young women affected by an early cervical cancer. The worldwide experience of this surgical technique, and the fact that this technique has become recognized, allows us to confirm that after a radical trachelectomy, pregnancies are possible and there is no increase of oncological risk. The current indications of Dargent's operation are well defined: invasive cervical cancer $< 2$ cm in maximal diameter; usual histological types of cervical cancer; and a young patient wishing to preserve her fertility [26]. In the future, new indications will be developed in association with neoadjuvant chemotherapy.

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Chapter 21

# Results of conservative management of ovarian malignant tumors

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## Introduction

Conservative and functional surgery is increasingly used in surgical oncology. Its aim is to preserve organ functionality and to limit radical resections. The development of new surgical procedures in oncologic gynecological surgery is a perfect example of this evolution. Although radical surgery remains the gold standard for the treatment of ovarian and cervical cancer, a conservative approach can be considered in patients with early stage disease, in order to preserve their ovarian function and fertility. These procedures are proposed to selected patients, depending on the histological subtypes and prognostic factors. Ovarian cancers are classified as epithelial (including borderline and malignant tumors) and non-epithelial cancers.

## Borderline ovarian tumors

The definition of borderline ovarian tumors (BOT) is based on the histological characteristics of the ovarian tumor and not on peritoneal implants. Four characteristics are used to define BOT: (1) epithelial proliferation with the formation of a papillary configuration; (2) a definable demonstration of atypical epithelial activity; (3) mild or moderate nuclear atypicality (these three characteristics are essential to differentiate BOT from ovarian cystadenoma); and (4) the absence of stromal invasion (which distinguishes BOT from invasive carcinoma) [1, 2]. Peritoneal implants are associated with BOT in 10–40% of cases. They are either non-invasive in 80% of cases (without stromal invasion) or invasive in 20% of cases [3–8]. A non-invasive implant was defined as a glandular or papillary proliferation, devoid of stromal invasion. Non-invasive peritoneal implants can be subdivided into two types, the epithelial type (with predominantly epithelial components) and the desmoplastic type (in which the epithelial components are embedded in a predominantly inflamed, desmoplastic stroma). Invasive implants are defined as a proliferation in the peritoneum with stromal invasion [3, 4, 6]. If biopsies or resection of peritoneal implants are too superficial, the degree of invasion cannot be accurately determined, and such implants should be considered as "non-specified" implants. In order to avoid this drawback, large biopsies or resection of peritoneal implants should be performed during the surgical procedure [8]. The pathological examination is therefore crucial to confirm: (a) the diagnosis of BOT (and peritoneal implants); (b) to determine prognostic factors; and (c) to decide upon the optimal treatment. In order to carry out adequate sampling, at least one section per cm of the greatest dimension of the ovarian tumor and the totality of the peritoneal implants should be examined [9].

In patients with non-invasive implants, complete surgical cytoreduction of peritoneal lesions is the only treatment likely to improve survival. The prognosis of patients with non-invasive implants is good [8]. However, when peritoneal implants are invasive, lesions evolve into more aggressive disease in one third of the cases [5, 6, 8]. In such cases, adjuvant therapy should be discussed.

A new entity designated "micropapillary serous carcinoma" (MP) associating peritoneal implants with a borderline tumor was described 10 years ago, in order to identify a subgroup of patients with a poor prognosis. Tumors with an MP pattern are more commonly associated with invasive implants. In that study,

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the presence of an MP pattern was an adverse prognostic factor [9]. However, Eichhorn *et al.* reported that the evolution of patients with non-invasive implants associated with an MP pattern was similar to that of patients with non-invasive implants without an MP pattern [10]. The real prognostic impact of this entity continues to fuel debate.

## Modalities of conservative surgery and clinical outcomes

The standard treatment of BOT consisted of a total abdominal hysterectomy and bilateral salpingo-oophorectomy, peritoneal cytology, omentectomy and multiple peritoneal biopsies. These procedures allowed us to perform adequate staging and to eventually propose adjuvant therapy exclusively to patients with invasive peritoneal implants. The prognosis of BOT is excellent. However, late recurrences (after 5 or 10 years) may occur [11]. Conservative surgery is defined as preservation of the uterus and at least a part of one ovary, in order to preserve fertility. BOT arise in a young population, where fertility is a major issue. The analysis of conservative management data is crucial in such patients. An analysis of the literature regarding the conservative management of BOT is difficult, because most of the series are retrospective and the duration of follow-up is too short (<5 years) to accurately evaluate the exact recurrence rate. Furthermore, the percentage of patients who were adequately staged varied among the series and depended on the treatment center. These variable numbers could account for the differences in recurrence rates. Three recent reviews were published concerning the results of conservative surgery in such tumors [12–14]. The risk of relapse, estimated at between 0 and 25%, is increased after this type of surgery [12–14]. It is also greater after a cystectomy (between 12 and 58% of cases) [12–14]. Some of these recurrences were observed long after treatment of the initial BOT (latest recurrences were 72 months in the series reported by Gotlieb *et al.* and 240 months in our series) [15, 16]. It is therefore highly likely that some of them are in fact new primary tumors, and not real recurrences of the initial BOT.

Lim-Tan published one of the first series on conservative surgery in BOT [17]. He reported on 35 patients (33 with stage I disease) treated conservatively [17]. In order to decrease the risk of recurrence after a cystectomy, Lim-Tan recommended a complete pathologic analysis of the margins to rule out any microscopic invasion [17]. Nevertheless, in the recent series reported by Papadimetriou, among 18 patients who underwent a cystectomy with histologically free margins, 7 relapsed [18].

In order to reduce the rate of relapse on the remaining ovary, some authors propose initial complete staging surgery with routine biopsies of the spared ovary. In our series, we performed this procedure in 14 patients with a macroscopically normal ovary. We found no tumor implants. Like Bostwick and Tazelaar, we observed only one relapse on the macroscopically normal ovary, that was routinely biopsied [19, 20]. It is important to note that this is not a harmless procedure, as it may induce infertility because of postoperative ovarian adhesions. Macroscopic inspection appears to be sufficient. Biopsies should be performed only in case of macroscopically suspicious lesions.

The high recurrence rate implies that the optimal treatment in patients with an intraoperative diagnosis of BOT, is a unilateral adnexectomy, which reduces the risk of relapse. A cystectomy should be performed only in cases of bilateral tumors and/or in patients with only one ovary (a previous history of adnexectomy). In case of a recurrent BOT on the remaining ovary, further conservative management (cystectomy) may be proposed to these patients, in order to preserve fertility. In our series, eight patients with recurrent BOT were reoperated on conservatively for their recurrent disease [16]. Six pregnancies were obtained in five of these patients [16, 21]. In the series by Gotlieb *et al.*, three cases of conservative management of recurrent BOT were reported [15]. Those 3 patients were alive after 6 months, 2 years and 7 years of follow-up [15].

## Survival of patients after conservative surgery

Does this increased risk of relapse affect the survival of patients? Zanetta *et al.* reported on a series of 189 patients who underwent fertility-sparing surgery [22]. Seven cases of progression into invasive ovarian carcinoma were observed, one of them very shortly (9 months) after the initial treatment of the BOT [22]. Six of these patients were alive after treatment of their recurrence [22]. In the recent series reported on by Park *et al.* among 164 conservatively treated patients with a median follow-up of 70 months, 9 recurrences were observed (with only one invasive recurrence in

the lung and pericardium, 82 months after the initial treatment) [23]. This latter patient died of her recurrent disease [23].

In the literature, among 1500 cases of conservative surgery in BOT, nearly 10 cases of patients treated conservatively for early stage disease developed a recurrence in the form of invasive carcinoma [24]. "Invasive" recurrent disease is therefore a rare event in patients with early stage disease. Most recurrent lesions were BOT with an excellent prognosis. Such major data explain why this increased recurrence rate following conservative surgery finally had no impact on survival [15, 21–23, 25–28]. Conservative surgery can therefore be safely performed in young patients treated for BOT and carefully followed up.

Nevertheless, it is not "scientifically" possible to affirm that there is no potential oncological risk after conservative management of BOT. Several series compared outcomes and survival after conservative and radical treatment of BOT [15, 21–23, 25–28]. However, such a comparison is very difficult because, in most cases, patients treated radically had more adverse prognostic factors (particularly advanced stage disease). Furthermore, if there is a potential risk for survival, it must be very small and only series including a very large number of patients treated conservatively with prolonged survival could explore such an important question. Only two large series of conservative treatment have actually been published: the Zanetta *et al.* series mentioned above and the recent series by Park *et al.* involving 189 and 164 cases [22, 23]. This high number of patients is nevertheless not sufficient to address the issue of a difference in survival between radical and conservative treatment of BOT.

## Limits of conservative surgery

Conservative management could be safely offered to most patients with early stage disease. However, in which subgroup of patients does such a procedure constitute a potential (small) oncological risk? It is very difficult to answer to this question because we have no specific data on this topic. Nevertheless, three different potential limits should be considered:

### Disease stage

Data in the literature concerning conservative management of BOT with peritoneal implants are rare (Table 21.1) [22, 29–32]. To date, only two large series have been published. These two series concluded that conservative management may be proposed to patients with peritoneal implants, providing these implants are entirely removed, with a reliable pathologic interpretation [22, 32]. Zanetta *et al.* reported on 12 patients with non-invasive implants treated conservatively: 3 of them developed a recurrent BOT in the spared ovary and 1 had progression to invasive ovarian carcinoma, but all 3 were salvaged with surgery and chemotherapy. All these patients were free of disease when this paper was written [22]. We recently reported on a series of 41 patients treated conservatively for a BOT with peritoneal implants [32]. Twenty-two recurrences occurred most of which were non-invasive. Nonetheless, one patient died of this recurrence [32]. The risk of lethal outcomes is therefore rare when serous BOT with non-invasive peritoneal implants are treated conservatively. Complete resection of implants is crucial in this context.

Yet is it possible to propose this surgical management to patients with invasive peritoneal implants? Zanetta *et al.* reported on seven patients with invasive implants treated conservatively [22]. Five BOT recurrences were observed in the spared ovary, but all patients were salvaged with surgery and were alive [22]. In our experience, we performed this treatment in three patients with invasive implants, one of whom had progressive peritoneal disease [32]. In the series reported by Prat and De Nictolis the only patient treated conservatively for BOT with invasive implants died of recurrent disease [29]. Considering the aggressiveness and the poor prognosis of BOT with invasive peritoneal implants, it seems judicious to propose conservative therapy exclusively to patients with BOT and non-invasive implants [32].

### Histological subtypes of the ovarian tumor

When we examined in detail the data of patients with early stage disease who developed a recurrence in the form of adenocarcinoma, the majority of them develop a mucinous tumor. Four out of five patients who died (of recurrent disease) after conservative management of early stage disease had a mucinous tumor [22–25]. This could be explained by the fact that most of these tumors were large and the histological analysis of such bulky tumors is difficult.

### Presence of a serous tumor with an MP pattern

The last potential limit concerns the presence of a serous tumor with an MP pattern. Only one series

**Table 21.1** Literature review of recurrence rates in advanced stage serous ovarian borderline tumor (in particular with invasive implants) after conservative treatment

| | No. | No. of recurrences | No. of deaths | No. of invasive implants | Recurrence in patients with invasive implants | Death in patients with invasive implants |
|---|---|---|---|---|---|---|
| Zanetta *et al.* [22] | 25 | 10 | 0 | 7 | 5 | 0 |
| Prat and De Nictolis [29] | 10 | 3 | 1 | 1 | 1 | 1 |
| Longacre *et al.* [30] | 21 | 5 | 0 | – | – | – |
| De Iaco *et al.* [31] | 21[a] | 4 | ? | ? | ? | ? |
| Uzan *et al.* [32] | 41 | 22 | 1 | 3 | 2 | 0 |
| **Total** | **97** | **40 (41.2%)** | **2 (2%)** | **11** | **8** | **1** |

[a] Mixed histology (mucinous, serous tumor).

was published on the conservative management of BOT with MP features. It included 15 cases (8 stage I and 7 stage III disease) [33]. Eleven recurrences were observed: six of them exclusively on the ovary, three exclusively on the peritoneum (invasive peritoneal disease in one) and two on the ovary and peritoneum. One of the last two patients succumbed to the recurrence (in the form of invasive adenocarcinoma). The other patients were actually disease free. Five patients achieved eight spontaneous pregnancies [33]. This recurrence rate might appear to be high but should be correlated with the high rate of bilateral ovarian involvement (2/3 patients) and with the fact that half of the patients had peritoneal implants. To date, while awaiting further publications on this topic, there is no indication for systematic radical treatment in patients with an MP pattern (except in cases with invasive peritoneal implants).

## Fertility results after conservative surgery

Pregnancies have been reported in patients with conservatively treated BOT. Lim-Tan *et al.* initially reported on eight patients who conceived [17]. Eight series (involving >10 patients desiring pregnancy) specifically reported the obstetrical results of conservative treatment [15, 16, 22, 23, 27, 28, 34, 35]. The rate of spontaneous pregnancies ranged between 30 and 80%.

Nevertheless, in spite of conservative management in BOT, some of these patients will experience infertility. Can ovarian stimulation or in vitro fertilization (IVF) be proposed to these patients, given that a number of studies incriminate hyperstimulation in the onset of BOT and ovarian cancer? In vitro data suggest that gonadotropins and/or a high dose of estrogens do not induce the proliferation of cell cultures from BOT [36].

Clinical data in the literature concerning this concrete case are rare (Table 21.2) [28, 32, 34, 37–46]. The largest multicentric experience in 30 patients previously treated conservatively for BOT and who underwent IVF procedures (or "simple" ovarian stimulation) was reported by Fortin *et al.* [45]. Thirteen pregnancies were reported and four recurrences (all of them in the form of borderline disease) [45]. It therefore seems possible to propose hyperstimulation to patients with stage I BOT without affecting the patient's prognosis. However, we think that the number of stimulation cycles should be limited, in order not to increase the potential risk of recurrence. Data in the literature on the safety of hyperstimulation in patients with peritoneal implants exclusively concern case reports (Table 21.2). It therefore does not seem possible to propose guidelines concerning hyperstimulation and IVF in these patients, even though some successful cases have been reported.

Bilateral salpingo-oophorectomy should be performed in patients with bilateral massive BOT and/or a recurrent BOT on the remaining ovary, and in whom preservation of a part of one ovary is unfeasible. Pregnancies have been reported in patients who underwent a bilateral salpingo-oophorectomy (with uterine preservation) for BOT, from donated oocytes

**Table 21.2** Literature review of cases of ovarian induction or in vitro fertilization (IVF) procedures in patients with a previous history of a borderline ovarian tumor

| Series | No. of patients | Ovarian induction (no.) | No. of IVFs | No. of stages II/III | No. of pregnancies | No. of recurrences (after stimulation) |
|---|---|---|---|---|---|---|
| Nijman *et al.* [37] | 1 | 0 | 1 | 1 | 1 | 0 |
| Mantzavinos *et al.* [38] | 2 | 0 | 2 | 2 | 1 | 0 |
| Hershkovitz *et al.* [39] | 2 | 1 | 1 | 1 | 2 (1 spontaneous after IVF) | 0 |
| Hoffman *et al.* [40] | 1 | 0 | 1 | | 1 | 0 |
| Morris *et al.* [34] | 6 | 4 | 2 | ? | 4 | ? |
| Beiner *et al.* [41] | 7 | 0 | 7 | 2 | 5 | 2 |
| Attar *et al.* [42] | 1 | 0 | 1 | 1 (III C micropapillary) | 1 | Rapidly progressive peritoneal disease |
| Fasouliotis *et al.* [43] | 5 | 0 | 5 | 0 | 6 | 1 |
| Fauvet *et al.* [28] | 11 | 6 | 5 | 1 | 3 | ? |
| Marcickiewicz and Brannstrom [44] | 3 | 0 | 3 | 0 | 2 | 0 |
| Fortin *et al.* [45] | 30 | 3 | 27 | 8 | 13 | 4 |
| Park *et al.* [46] | 5 | 0 | 5 | 1 (IIIC nodal) | 5 | 0 |
| Uzan *et al.* [32] | 8 | 3 | 5 | 8 | 5 | 3 |

or the successful transfer of frozen embryos, obtained before bilateral salpingo-oophorectomy [47–49]. Cryoconservation of ovarian tissue could also be proposed in such cases, but no pregnancies have been obtained under such conditions [50].

Patient follow-up is based on a clinical examination and abdomino-pelvic and vaginal ultrasonography. A paper on this topic by Zanetta *et al.* failed to demonstrate the value of using blood markers in this context but, pragmatically, we use them combined with two other procedures [51].

Should we reoperate patients to remove the remaining ovary when fertility is no longer an issue for those who have conceived after conservative treatment of a BOT? No standard practice exists in the literature regarding this question. Although the recurrence rate is between 0 and 25%, routine oophorectomy appears to be useless in 75–100% of cases [14]. Furthermore, these recurrent lesions (mostly BOT) could easily be cured, using a simple surgical procedure. In our institution, the systematic removal of the spared ovary is thus not mandatory provided patients are followed up regularly. Oophorectomy is then proposed exclusively for relapses. However, some patients prefer to undergo an oophorectomy after achieving a pregnancy, for psychological reasons.

# Epithelial ovarian cancer

## Indications for conservative surgery

The differential criterion between epithelial ovarian cancer (EOC) and BOT is the invasion of the ovarian stroma. The standard surgical procedure for EOC is a radical hysterectomy with bilateral salpingo-oophorectomy. The results concerning conservative management of EOC are difficult to analyze in the literature, because many of the published series are either mixed dealing with conservative treatment in epithelial and non-epithelial ovarian cancer, or including invasive and borderline ovarian tumors and considering them as epithelial lesions. Some studies reported on the results of conservative management but epithelial, borderline and non-epithelial tumors were all included. Only a few studies (5 series [>10 cases]) have focused on conservative treatment exclusively in EOC (Table 21.3) [52–57]:

- Colombo in 1994 and Zanetta in 1997 published the first series specifically devoted to EOC. These series included 56 patients [52, 53].
- An American multicenter study (8 different centers in USA) including 52 cases was reported in 2002 [54].

**Table 21.3** Literature review of results conservative management in epithelial ovarian cancer (5 series reported including >10 cases)

| | Stage IA grade 1 | Stage IA grade 2 | Stage IA grade 3 | Stage IC grade 1 | Stage IC grade 2 | Stage IC grade 3 |
|---|---|---|---|---|---|---|
| **Italian series** Zanetta *et al.* /Colombo *et al.* [52, 53] | 1 recurrence out of 24 patients | 3 recurrences out of 8 patients | 1 recurrence out of 4 patients | No recurrence out of 10 patients | 1 recurrence out of 6 patients | No recurrence out of 3 patients |
| **American series** Schilder *et al.* [54] | 2 recurrences out of 33 patients | 2 recurrences out of 6 patients | No recurrence out of 3 patients | No recurrence out of 5 patients | 1 recurrence out of 3 patients | No recurrence out of 2 patients |
| **French series** Morice *et al.* [55] | 1 recurrence out of 13 patients | 4 recurrences out of 14 patients | 1 recurrence out of 3 patients | 2 recurrences out of 2 patients | No patient | 1 recurrence out of 1 patient |
| Park *et al.* [56] | 1 recurrence out of 29 patients | No recurrence out of 3 patients | 4 recurrences out of 4 patients | 1 recurrence out of 15 patients | 1 recurrence out of 2 patients | 2 recurrences out of 2 patients |
| Anchezar *et al.* [57] | 1 recurrence out of 10 patients | No patient | 1 recurrence out of 1 patient[a] | No recurrence out of 3 patients | No recurrence out of 1 patient | No recurrence out of 1 patient |
| **Total** | **6 recurrences out of 109 patients** | **9 recurrences out of 31 patients** | **7 recurrences out of 15 patients** | **3 recurrences out of 35 patients** | **8 recurrences out of 12 patients** | **3 recurrences out of 9 patients** |

[a] Patient considered as having a stage IA grade 3 tumor after pathological review of the initial tumor at the time of the recurrence.

- A French multicenter study was published on a series of 34 patents with EOC with strict inclusion criteria (a systematic review of slides, complete staging surgery and chemotherapy for patients with stage ≥IC-55).
- The largest series was recently published by Park *et al.* in 2008 with inclusion criteria very close to those of the French series [56].
- A recent series from Argentina reporting 18 patients [57].

Initially, Di Saia proposed conservative treatment for EOC, but with selected inclusion criteria (i.e. patients who desire fertility; who are willing to undergo close gynecological follow-up and a stage IA; well-encapsulated ovarian cancer without peritumor adhesions and without involvement of the ovarian surface; and/or no mesovarium infiltration and negative peritoneal washings) [58]. The histological type plays a major role among inclusion criteria. Thus, only serous, mucinous and endometrioid EOC can be considered for conservative management even if a recent Asian paper suggested that such management could be proposed for clear cell tumors [59]. While awaiting further studies concerning such histological subtypes, patients with clear cell and anaplastic EOC should not be considered for conservative treatment, because of the high risk of relapse on the remaining ovary.

The results reported in those four studies suggested that conservative surgery could be safely performed in patients with stage IA grade 1 (and probably grade 2) disease (Table 21.3). In 15 patients with stage IA, grade 3 disease, 6 recurrences were observed (Table 21.3). Consequently, conservative management should not be performed in such cases.

Stage IC disease was the subject of heated debate because of conflicting results in the five published series (Table 21.3). Clarifying the criteria used to classify patients with stage IC disease in those different series could potentially explain the differences between the series. In the 1988 FIGO classification, patients are classified as having stage IC disease in case of a uni- or bilateral tumor with: (a) tumor spread on the surface of the ovary (excrescences); and/or (b) ascites containing malignant cells or positive cytology after positive washing; and/or (c) capsular rupture during the morphologic analysis. Thus, patients included with stage IC disease were probably "dissimilar" in terms of the criteria used to classify disease as stage IC. Furthermore, the histological subtype (mucinous, serous or other) was perhaps somewhat different in those four series concerning this substage

of disease. Such fine differences could explain the absence of homogeneity in the literature. If we take into account the recent data from Park *et al.*, conservative management could probably be considered in stage IC, grade 1 disease but should not be performed for grade 2 or 3 disease [56].

A recent paper using the analysis of the Surveillance, Epidemiology and End Results (SEER) database suggests the absence of deleterious impact on survival of preserving the ovary in stage IA or IC disease [60]. As stated by the authors, "to detect a 20% difference in survival for patients with stage IC disease, a cohort of 1282 pts with 52 deaths is required" [60]. Thus, as none of the published series included such a large number of patients, it is not possible to draw definitive conclusions about the safety of conservative management in this setting. In the recent paper from Schlaerth *et al.*, the survival of 20 patients treated conservatively for a stage I disease, is similar to the survival of patients (<45 years) treated radically [61]. Nevertheless, such comparison is somewhat "inadequate" because the prognostic factors, even if none of them reach the level of the statistical significance, had a trend to be "better" in the group of patients treated conservatively [61]. Conservative surgery should not be proposed to patients with disease exceeding stage I [55–57].

In patients with a "limited" indication for conservative surgery (stage IA grade 3 disease, stage IB or IC grade 2 or 3 disease) an alternative option could be considered: the removal of both ovaries but with uterine conservation (without uterine curettage at the time of staging surgery) to preserve a possibility of "fertility" (oocyte donation or another procedure). This option has never been explored in EOC but should be evaluated. In the recent SEER database analysis, there was no impact on survival of uterine preservation in stage IA or IC disease [60].

The prognosis of patients with recurrent EOC after conservative surgery remains poor, particularly when recurrent disease arises outside the preserved ovary [62].

## Surgical procedure for conservative surgery

This conservative surgery should only be considered after adequate surgical staging. This staging should include peritoneal washings, excision of any suspicious peritoneal lesions, multiple peritoneal biopsies, omentectomy and endometrial curettage. A pelvic and paraaortic lymph node dissection is usually discussed in early stage disease, particularly in the case of mucinous tumors [63].

Munnell proposed a systematic biopsy of the remaining ovary [64]. He also considered that contralateral microscopic involvement existed in 12% of EOC [63]. However, systematic biopsies of contralateral ovarian cancer can induce infertility by provoking postoperative adhesions on the remaining ovary. Moreover, many authors did not find any microscopic implants in the macroscopically normal ovary [53, 55]. Yet Benjamin *et al.* found microscopic disease in the contralateral ovary which was macroscopically normal in 3 patients (2.5%) in their series of 118 patients with stage I EOC [65]. However, these 3 patients had a grade 3 tumor and none of the patients with stage I, grade 1 or 2 disease had occult metastasis on the contralateral ovary [65]. Consequently, we do not recommend routine biopsies of the contralateral ovary if preoperative vaginal ultrasonography did not reveal deep parenchymous abnormalities in the initially undiseased contralateral ovary and if it appears to be macroscopically normal during the surgical procedure.

## Fertility results following conservative treatment of EOC

Few fertility results are available in the literature. Zanetta *et al.* obtained 27 pregnancies in 20 patients [52]. In the American series, 17 pregnancies were reported in 24 patients attempting to conceive [54]. In the French series, only 9 pregnancies were achieved and Park reported on 15 pregnancies in 19 patients [55]. In the series reported by Anchezar *et al.*, 7 pregnancies were reported in 6 of the 7 patients who attempted to conceive [56]. In the case of persistent infertility, ovarian stimulation or IVF continues to be contraindicated.

Patient follow-up is based on a clinical examination, blood markers and the use of systematic imaging (abdomino-pelvic ultrasonography).

Recourse to completion surgery after childbearing (or after the age of 40 in patients who fail to become pregnant) is still debated. However, a case of a recurrent EOC 10 years after conservative treatment, could suggest discussing the removal of the remaining ovary, in order to reduce the risk of recurrence on the spared ovary.

**Table 21.4** Literature review of fertility results following conservative management in germ cell tumors (series published after 1995)

| Series | Peccatori *et al.* 1995 [66] | Mitchell *et al.*[a] 1999 [67] | Brewer *et al.*[b] 1999 [68] | Tewari *et al.* 2000 [69] | Low *et al.* 2000 [70] | Zanetta *et al.* 2001 [71] | Tangir *et al.* 2003 [72] | Zanagnolo *et al.* 2004 [73] | Boran [b] *et al.* 2005 [74] | Ayhan[a] *et al.* 2005 [75] | Kang[b] *et al.* 2008 [76] | De La Motte Rouge[a] *et al.* 2008 [77] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. patients | 129 | 69 | 26 | 72 | 74 | 169 | 106 | 55 | 23 | 29 | 20 | 52 |
| No. conservative management | 108 | 50 | 16 | 46 | 74 | 138 | 64 | 39 | 23 | 15 | 15 | 41 |
| Menstration maintained | ? | 24/26 | 14 | ? | 43/45 | 128/130 | 32/40[c] | 26 | 19/23[c] | 10[d] | 15 | 39/40 |
| No. pregnancies | ? | 11 | 5 | ? | 19/20 | 55 in 32 patients | 38 in 29 patients | 11 | 6 in 5 patients | 3 | 2 | 19 in 12 patients |
| No. conservative management, stages II/III/IV | 37 | ? | ? | 1[e] | 19 | 46 | 11 (9 pregnancies) | 11 | 8 (4 pregnancies) | ? | ? | 4 pregnancies |

[a] Papers reporting exclusively on endodermal sinus tumor or non-dysgerminomatous tumors.
[b] Paper reporting only on dysgerminomatous tumors.
[c] Menstruations considered as similar to those observed before chemotherapy.
[d] Five patients excluded from assessment of menstruations because of a lethal recurrence.
[e] Pregnant patient.

## Non-epithelial ovarian cancer

Non-epithelial malignant tumors are characterized (compared to epithelial cancers) by: (1) the occurrence of disease in younger patients; and (2) an (overall) good prognosis of this tumor (even in case of extra-ovarian disease) as in most cases the chemo-curability of these tumors is excellent. They could be classified into two main groups: malignant germ cell tumors (MGCT) and sex cord stromal tumors (SCST).

### Malignant germ cell tumors

Most of the papers concerning the results of conservative surgery in non-epithelial cancers, concern this group of tumors (Table 21.4) [66–77]. The most frequent lesions in this group are dysgerminomas, endormal sinus tumors (EST), malignant teratoma and mixed subtypes. The type of chemotherapy administered against such tumors is the "BEP" regimen (bleomycin, etoposide and cisplatin). Conservative surgery is the standard management in young patients. Staging (nodal or peritoneal) procedures are discussed in this context. In non-dysgerminomas with a macroscopically normal ovary, biopsies of the contralateral ovary are not recommended. In dysgerminomas, this procedure could be considered because there is a potential risk of occult disease in 10% of cases. For example in the recent series by Boran *et al.*, 2 out of 17 patients (11%) with a macroscopically normal contralateral ovary had occult involvement [74].

The fertility results in the series reported after 1995 are shown in Table 21.4. Menstruation and endocrine ovarian function were maintained in a very large majority of these young patients treated with the BEP regimen (Table 21.4). Conservative management of a part of one ovary could be considered in patients with bilateral involvement (in the case of teratomas) or in patients with peritoneal disease treated with adjuvant chemotherapy (particularly in dysgerminomas or malignant teratoma) (Table 21.4).

Given the high curability rate among these patients, no completion surgery is discussed after childbearing.

### Sex cord stromal tumors

The most frequent subtypes of these tumors are granulosa cell, Sertoli–Leydig and thecal cell tumors. Very few papers are devoted to the conservative management of such tumors (mostly case reports or short series). In the series by Zhang *et al.*, among the 376 women treated for SCST, 71 young patients underwent uterine preservation for stage I disease [78]. The survival of patients treated conservatively and radically was similar [78].

Two important characteristics observed in granulosa tumors exert an impact on conservative surgery: bilaterality is uncommon (between 2 and 8% of cases [79] and these tumors are frequently associated with endometrial disorders (hyperplasia or cancers). Consequently, random biopsies of the contralateral ovary are not required (if macroscopically normal) but uterine curettage should be systematically performed. The overall prognosis of granulosa cell tumors is good in early stage disease (stage IA) and conservative management could be considered in young patients with a similar stage. However, conservative management should not be proposed for higher stages (or in the case of ovarian capsule rupture during initial surgery) because the prognosis is less clear cut.

The use of completion surgery after childbearing continues to be debated in SCST [79].

## Conclusions

Conservative treatment yields good fertility results and does not affect the survival of patients with borderline ovarian tumors. It should be considered for young women desiring fertility, even if peritoneal implants are discovered at the time of initial surgery. In case of infertility, medically-assisted procreation techniques may be proposed to patients with stage I BOT with a limited number of stimulation cycles.

In patients with epithelial ovarian cancer, conservative surgery of an ovary and the uterus can only be considered in adequately staged patients, with a stage IA, grade 1 (and probably 2) serous, mucinous or an endometrioid tumor and careful follow-up. Such management could probably also be safely proposed for stage IC, grade 1 disease.

In patients with non-epithelial ovarian cancer, conservative surgery is highly applicable, particularly in patients with malignant germ cell tumors.

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Chapter 22

# Embryo cryopreservation as a fertility preservation strategy

Pedro N. Barri, Anna Veiga, Montserrat Boada and Miquel Solé

## Introduction

Survival rates after cancer have increased significantly in recent decades; however, these treatments also have drawbacks and patients (or parents in the case of children) must be informed of the long-term side effects of oncological treatments and the possible options for preserving the fertility of these patients. It is important to set out clearly the possible risks of developing ovarian failure or azoospermia with oncological treatments. These will depend on the age of the patients and on the type, dose and duration of chemotherapy, and on the field, dose and duration of radiotherapy.

The strategy must be individualized in each case depending on:

- Patient's age.
- Type and stage of the cancer.
- Therapeutic plan to be followed.
- Foreseeable long-term effects.
- Possibility or impossibility of delaying the start of treatment.
- Whether or not the patient has a partner/spouse.
- Biology of the tumor and potential for metastasis in ovary.

If ovarian stimulation is possible, embryo cryopreservation is the method with the greatest chance of success so far. However, it is only possible if there is a partner or if the patient accepts donor sperm. It is very important to clarify which are the safest ovarian stimulation protocols to be used in these patients, although current data suggest that in certain cancers a cycle of ovarian stimulation does not increase the risk of recurrence.

Since the first pregnancy obtained from cryopreserved human embryos [1], the yield and safety of embryo cryopreservation programs have improved notably with successful freezing of zygotes and cleaving of embryos and blastocysts, and pregnancies are regularly obtained after thawing and transfer of embryos in any of these stages. Our first pregnancy after transferring a previously frozen embryo was obtained even though only one of the embryo's original four cells survived thawing and was transferred [2].

## Outcome of embryo-freezing programs

Embryo cryopreservation is the method of choice and with the greatest chance of success; however, it can only be applied if there is a male partner or if the couple accept donor sperm.

It is accepted that 15–20% of the 3 million children born after in vitro fertilization (IVF) were conceived following the transfer of embryos that had previously been frozen and thawed. That means that these 500 000 or so children make up a wide population that proves the efficacy and the safety of embryo cryopreservation [3, 4].

Nevertheless, cryopreservation affects the potential for embryo implantation, which is lower than for fresh embryos. It is important to choose the freezing protocol among the slow-freezing methods or those that use vitrification, just as it is also important to choose the stage at which the embryo is to be frozen. Zygotes are normally frozen on 2PN, cleaving embryos or blastocysts, with comparable rates for survival after thawing and for pregnancy [5].

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**Table 22.1** Institut Universitari Dexeus embryo freezing program: 1987–2004

| | 20 318 embryos thawed | | | | |
|---|---|---|---|---|---|
| Cycles | Transfers | Thawed embryos | Survival | Pregnancy/transfer | Babies born |
| 6715 | 5354 | 20.318 | 66% | 22.5% | 807 |

**Table 22.2** Institut Universitari Dexeus Embryo freezing program ($n = 2547$ transfers) Results according to frozen time (years)

| Frozen time | Pregnancy rate/transfer | |
|---|---|---|
| 5–7 years | 16/44 | 36.4% |
| 7–9 years | 5/16 | 31.3% |
| ≥~9 years | 5/8 | 62.5% |
| **Totals** | | |
| **≤ 5 years** | **853/2479** | **33.68%** |
| **> 5 years**[a] | **26/68** | **38.2%** |

[a] One baby born after 10 years and 1 baby born after 14 years of cryopreservation.

At the Instituto Universitario Dexeus, Spain, we use zygote-freezing only in cases of fertility preservation in oncological patients or in some egg donation cycles where it is not possible to synchronize the donor's cycle with that of the recipient. An analysis of our results for zygote-freezing in the period 2002–2007 showed that survival rates were 86.5% with pregnancy rates per transfer of 41.1%. It is important to check embryo viability after thawing by cultivating the embryos overnight to confirm that they have started the first embryonic divisions. Freezing cleaving embryos gave rather lower survival rates of around 70%. Similar rates were achieved by freezing blastocysts and in these cases the post-thawing viability was evaluated at 4 h to check blastocyst expansion [5–9].

In our experience, with our embryo-freezing program during the period 1987–2004, and with 20 318 embryos thawed, we had a global survival rate of 68%. A total of 5354 cryoreplacements were performed leading to the birth of 807 children and with a birth-per-cryoreplacement rate of 22.6% (Table 22.1). One important aspect to bear in mind when this technique is applied to oncological patients is to analyze the results according to the time that the embryos have been frozen. Our data show that this variable is not relevant as the pregnancy rates obtained following cryoreplacement before and after 5 years of freezing do not differ (Table 22.2).

Recently vitrification has been developed for both oocyte and embryo cryopreservation. The results so far seem to show higher post-thaw survival rates and higher rates of development to the blastocyst stage [10, 11]. Implantation and clinical pregnancy rates are within the normal range. As far as safety is concerned, studies on the neonatal outcome after embryo vitrification have shown no increase in the congenital malformation rate with a neonatal outcome comparable to fresh embryo transfers [3, 12, 13].

Another point to take into account in these cases is the analysis of the patients' preferences regarding future use of the embryos if the patient should die. In such cases, Spanish law allows the patients to decide between anonymous donation of the embryos to other couples, donation for research or destruction of the embryos. We feel that the patients' freedom of choice must be respected with explanations of all the possibilities being given before unfavorable circumstances arise that could condition their decision [14, 15].

## Ovarian stimulation protocols for IVF and embryo cryopreservation in oncological patients

In oncological patients two special circumstances often arise: a short time to stimulate ovulation and the necessity of not reaching high estradiol levels. It is also important to start the ovarian stimulation before the chemotherapy as the results of the IVF cycle will be very poor if a course of chemotherapy has been performed [16].

In these cases, ovarian stimulation can be done using aromatase inhibitors in combination with gonadotropins. Tamoxifen was the first drug used in these protocols but letrozole soon proved to be just as safe and much more effective [17]. Letrozole is a powerful third-generation aromatase inhibitor; with a half-life of 48 h it significantly suppresses plasma estradiol levels. Data from recent publications suggest that letrozole is more effective than tamoxifen in ovulation stimulation protocols for oncological patients [18].

With regard to the gonadotropins, it seems to be better to use recombinant follicle stimulating hormone (FSH) preparations devoid of luteinizing hormone (LH) to limit the estradiol levels that will be reached during stimulation. To prevent early luteinization or premature ovulation it is fundamental to combine these drugs with a gonadotropin-releasing hormone (GnRH) -antagonist analogue so that we can act at any point in the menstrual cycle.

There is now evidence that the survival of oncological patients who have followed an ovarian stimulation protocol for an IVF cycle, and the freezing of any embryos that are obtained, is identical to that of patients who do not undergo this protocol [17].

If the oncologist authorizes a 2–3-week delay in the treatment, we will start treatment with GnRH antagonists at any point in the cycle and will start stimulation when plasma estradiol is below 50 pg/ml. After this, the patient will begin treatment with 5 mg/day of letrozole for 5 days, combining this treatment with the daily administration of 150 IU of recombinant FSH. The ovulatory discharge will be with a bolus of GnRH agonist, and letrozole or antagonist treatment will be maintained following follicular aspiration.

## Results of embryo cryoreplacements in oncological patients

The first cases of embryo freezing in oncology patients took place more than 10 years ago, with the application of a natural IVF cycle with embryo cryopreservation prior to chemotherapy for carcinoma of the breast [19]. However, the first pregnancies were published some years later with a case of embryo cryopreservation after diagnosis of stage IIB endometrial cancer and subsequent pregnancy in a gestational carrier [20].

Recently several pregnancies have been published in oncology patients who had frozen their embryos before starting chemotherapy [14–18]. In our experience, we had a birth of a healthy boy 4 years after embryo cryopreservation was carried out in a patient with a bilateral borderline ovarian tumor who was treated initially with an IVF cycle in which 4 embryos could be frozen. Conservative surgery of the ovarian tumor was practiced later and at 3 years the embryos that survived freezing and thawing were cryoreplaced. A clinical pregnancy was obtained that led to the birth of a healthy boy. The improved results from vitrification of oocytes means that now some oncology patients have opted for this strategy and the first births achieved in this way have been published [21].

In applying embryo-freezing techniques to preserve the fertility of oncology patients, it is very important to know the couple's preference for the disposition of any unused embryos. Couples are normally offered the possibility of choosing, in the event of death or unforeseen circumstances, between the destruction of the embryos, donation for research, anonymous donation to other infertile couples or leaving the decision to the surviving partner. The oncology patients make similar choices to the infertile patients who undergo IVF with regard to donation of the embryos for research or to other infertile couples, but most oncology patients reject destruction of the embryos and accept this option in a much lower percentage than normal IVF patients [14].

## Conclusions

Up to now, embryo cryopreservation has been the only clinically accepted method for preserving the fertility of oncology patients before they undergo chemotherapy and/or radiotherapy. The post-thawing pregnancy rates are acceptable and are around 30% per cryoreplacement depending on the number of embryos available and their quality.

However, this option does have some drawbacks such as:

(a) It is not feasible for pubertal girls.
(b) Ovarian stimulation may be contraindicated and oocyte retrieval and IVF may cause a delay in the initiation of oncological treatment that may not be acceptable in some cases.
(c) Spermatozoa from a male partner or from a donor is required.
(d) Possible religious or moral objections.

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Chapter 23

# Oocyte cryopreservation

## Slow freezing

Andrea Borini and Veronica Bianchi

In the last 20 years significant improvements have been made in in vitro fertilization (IVF) procedures especially involving gamete cryopreservation. Several factors have driven the research in this field including the law (in Italy) and need for optimization of particular applications (like egg donation). Moreover, the possibility to freeze eggs is an important requirement to preserve fertility potential in patients at risk for fertility loss due to premature ovarian failure or invasive treatments like chemotherapy and/or radiotherapy. Ovarian cryopreservation presents a valid alternative to egg freezing in some circumstances. The possibility to store oocytes for a later use is also an important consideration for women who choose to postpone motherhood for personal or professional reasons.

The ability to freeze eggs as a routine IVF procedure is an important tool to optimize egg donation and allow for independent synchronization of donor and recipients, and quarantine of specimens for sexually transmitted diseases. Another fundamental benefit of egg cryopreservation is to avoid potential problems associated with legal status and ownership of cryopreserved embryos in the event of divorce.

## Chemical and physical aspects

Although the first human live birth from cryopreserved oocytes was reported more than 20 years ago [1], success rates in assisted reproductive technologies using frozen oocytes have lagged behind those using frozen embryos or blastocysts. Technically speaking, slow-freezing protocols were the only option available to cryopreserve oocytes for about 10 years, until development of vitrification over the last 5 years as an alternative to the original methods.

Any newly developed protocol should consider the biochemical and physical properties of the oocyte. Cell survival is intimately associated with the composition and permeability characteristics of the cell membrane, the surface to volume ratio of the cells and the difference in osmotic pressure between the two sides of the membranes [2, 3]. Moreover, the cytoplasm of the oocyte contains a high proportion of water in comparison to other cells; damage due to ice crystal formation in the phase transition of water to ice was an initial hurdle to overcome during freezing procedures. Protocols that include dehydration of oocytes before and/or during the cooling procedure reduce ice crystal formation and lead to improved clinical outcomes. Consequently, cryobiology studies analyzing the physical and chemical proprieties of cryoprotectant agents (CPAs) have been an important contribution to this field. The first fundamental studies were conducted by Arrhenius *et al.* and established an important connection between temperature, activation energy and the rate of chemical reactions, showing that long-term preservation was possible at very low temperatures due to decreased rates of all the biological reactions [4].

Later, Mazur *et al.* formulated a series of equations aimed to explain the multifactorial cellular damage due to freezing procedures [5, 6]. First, it was important to analyze the membrane properties of the cell, regarding permeability and internal osmotic pressure. Subsequently, it was necessary to monitor how these parameters changed during the cooling phase according to the freezing rate, decreased temperature, change in vapor pressure and solute concentrations during ice crystal formation. From these observations, Mazur established that a low cooling rate maintains an osmotic balance between the two sides of the membrane; however, the hypertonic milieu that surrounds the cell may cause irreversible damage to the membrane protein structures. Faster cooling rates, on the contrary, limit this situation but also prevent all the

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intracellular water from leaving the cell and, consequently, result in ice crystal formation and cell death. To partially overcome to this issue, CPAs have been introduced; in fact one role of CPAs is to protect biological materials against cooling injuries from ice crystals and high concentrations of solute during cryopreservation [7, 8].

Since the introduction of glycerol as a permeable cryoprotective agent in sperm cryopreservation by Polge *et al*., and the subsequent discovery and use of dimethyl sulfoxide (DMSO), many cells and tissues have been frozen but the cooling procedures were mostly theoretical [9, 10].

All freezing methods developed to date rely on the presence of one or more CPAs in molar concentrations.

In general cryoprotectants may be divided into two groups:

(a) Permeating agents such as glycerol, DMSO, ethylene glycol and 1,2-propanediol (PROH). All of which are chemically characterized as having a relatively low molecular weight and can penetrate the lipid bilayer of the cell with a slower permeability than water.
(b) Non-permeating agents that remain in the extracellular solution as a result of their size or polarity. These CPAs include sugars and macromolecules such as sucrose, Ficoll and raffinose, as well as proteins and lipoproteins.

Cryoprotective agents can reduce the toxic effects of high concentrations of other compounds in the solution [11], and their protective effects are related to their hydrophilic ability to create hydrogen bond and consequently to interact with water and to achieve high aqueous solubility.

With glycerol and PROH, the hydrogen bonding is between the hydrogen of the OH groups and the water. The oxygen on the DMSO molecules bind to the water protons with the release of heat. During slow cooling, when the cells are very dehydrated and are surrounded by concentrated salts, the cryoprotectants appear to reduce damage caused by the high levels of salt.

These CPAs, however, can have dramatic osmotic effects upon cells during the freezing/thawing procedures; when exposed to permeating solutes the oocyte undergoes extensive initial dehydration and shrinkage followed by a return to near the original volume as the cryoprotectant enters the cell and replaces the intracellular water. This causes a double flux across the membrane (the water exits the cell while the CPA enters) that influences both the intracellular solute concentration and the cell volume. The extent of shrinkage and swelling can cause damage or even cell death due to the osmotic stress acting on the oocyte membrane. To reduce stress from excessive swelling, non-permeating molecules are added to the CPA mixture. Sucrose is the most commonly used, but trehalose and choline have also been utilized in cooling protocols [12–14].

Usually, in the most widely adopted slow cooling protocols, cryoprotectant concentrations are around 1.5 M for the penetrating agent (mainly PROH or DMSO) and 0.1–0.3 M for the non-penetrating agent (usually sucrose).

The analysis of cell-volume change dynamics is important to evaluate approaches to improve freezing protocols. Addition of cryoprotectants can cause cell damage due to volume changes and chemical toxicity; optimal exposure should aim to minimize osmotic stress while avoiding chemical toxicity and allow sufficient permeation and dehydration to achieve protection from freezing injury.

Several authors approached those issues with different ideas: Yang *et al*. tried to increase cryoprotectant exposure temperature to achieve faster dehydration rates [15].

Quintans *et al*. adopted a stepwise addition of the permeating cryoprotectant (PROH) to reduce volume excursion [16], while Boldt *et al*. tried to use a sodium-depleted freezing medium combined with a lower seeding temperature to improve post-thawing recovery [17].

However, no measurement of the actual response of the cells to these changes was performed in these studies. To address this, Paynter *et al*. tried to improve the cryopreservation technique by evaluating several factors involved during the equilibration of oocytes to cryoprotectant interaction and decreased temperatures [18]. The authors measured the osmotic response by monitoring the oocytes for 10 min during a two-step addition of the permeating cryoprotectant PROH (0.75 and 1.5 M PROH). Following this, the oocyte osmotic response to 1.5 M PROH and 0.2 or 0.3 M sucrose was measured. Those authors found that each oocyte shrank during the first exposure to the cryoprotectant (0.75 M PROH) as water left the cell and then gradually re-expanded as water and cryoprotectant entered. The entire volume equilibration process took around 10 min to complete.

During the second exposure to an increased cryoprotectant concentration (1.5 M PROH) the oocytes

underwent the same phases of shrinkage and re-expansion, while in the last set of exposure experiments (1.5 M PROH plus sucrose) the shrinkage rate was faster with an obvious reduction of cell volume before freezing to avoid intracellular ice crystal formation.

This paper targeted the important concept of a shrinkage and re-expansion time frame in which oocytes can recover after PROH exposure.

An equally important consideration for oocyte survival involves the removal of permeating cryoprotectants. When a cell containing cryoprotectant is placed in medium with a lower concentration of cryoprotectant, water enters the cells to dilute the cryoprotectant at a faster rate than the cryoprotectant can leave the cells, which causes swell and potential burst. This problem can be overcome by controlled removal of the cryoprotectants in a series of decreasing concentration steps.

This does not prevent the cells from swelling, but reduces the corresponding magnitude and achieves the desired outcome of gradual CPA removal and rehydration in a controlled manner. Cells are moved from one solution to the next after they re-acquired their normal level of hydration.

An alternative method for removing cryoprotectants from cells is to use a high concentration of a non-penetrating molecule such as sucrose; this extracellular high concentration serves to balance the high intracellular cryoprotectant concentration as it reduces the difference in osmolarity between the intra and extracellular environments. The oocytes shrink proving that both the cryoprotectant and the water are leaving the cell reducing the intracellular volume. The use of high sucrose concentrations allows a one step thawing protocol that is simpler and faster than multi-step dilution procedures.

## Morphological variables

In addition to surviving the cryopreservation/warming process, the oocyte needs to maintain competence to fertilize and develop in vitro to the appropriate embryonic stage without any structural alterations.

It has been widely demonstrated that low temperatures and cryopreservation procedures in general may induce critical damage to the cell substructures, especially due to the peculiar characteristics of the metaphase II (MII) oocyte. At this maturational stage the chromosomes are aligned on the metaphase plate in strict relation to the meiotic spindle. This is a delicate structure responsible of the correct chromosome segregation during the maturational process that ends with extrusion of the second polar body. The meiotic spindle is a highly dynamic bipolar structure made of microtubules that have the ability to disassemble and reassemble under particular conditions. They are dimers composed of α and β tubulin, more dispersed at the microtubule organizing center (MTOC) and more compacted at both ends in contact with the kinetochore; the spindle has a typical barrel shaped structure with the chromosomes suspended within it.

Meiotic spindle is frequently abnormal in older woman both with regard to chromosome alignment and the microtubule structure, showing that the increase in the genetic abnormalities is often associated with poor structural quality of the egg [19]. This study was conducted on women undergoing natural cycles, thus avoiding bias related to ovarian stimulation or in vitro oocyte aging.

Another factor associated with meiotic spindle damage is temperature. Several studies using the mouse model showed that low temperature can cause depolymerization of the tubulin dimers and a transient or permanent loss of the spindle [20, 21].

In mouse, oocyte incubation at 25, 18 or 4°C can lead to microtubule disassembly with an increase of the monomeric tubuline, but within 1 h at 37°C the spindle is able to significantly reassemble [22].

Conversely, in the human, this structure is more sensitive to fluctuations and the rate of recovery is limited. If temperature is lowered from 37 to 27°C the spindle depolymerized within 5 min and can repolymerize only if this suboptimal condition is not extended beyond 20 min [20, 23].

During cryopreservation oocytes undergo more stressful situations, due to prolonged exposures to low temperatures leading to spindle alterations and a potential increase in chromosome segregation errors resulting in higher aneuploidy rates or even fertilization failure. It is also known that cryoprotectants play a key role in protecting this structure if the concentration and exposure time is appropriate [11].

Conflicting opinions have been expressed in which some authors reported that freezing procedures alter meiotic spindle organization [24] while others reported no significant damage occurred after freezing [25, 26]. Cytogenetic analysis performed for chromosomes 13, 18, 21 X and Y did not highlight an

increase in aneuploidies or chromosomal dispersion in embryos derived from fresh or frozen–thawed oocytes (28 versus 26%, respectively) [27].

In order to have a better understanding, several studies on recovery of the meiotic spindle post-thaw have been conducted using two approaches: the Polscope® and the confocal immunostaining microscopy, the latter undoubtedly offering the most detailed data on spindle structure but unfortunately requiring a fixation step that causes loss of oocyte viability. The Polscope® has been used instead as an alternative approach. It is a microscopy optical system that allows the observation of highly ordered subcellular structures, such as the spindle, through polarized light [28, 29]. It has been showed that microtubules are responsible for spindle birefringence and that spindle retardance is related to microtubule density [30]. This system is very important for routine application as it allows visualization of the spindle and the inner layer of the zona pellucida, and to roughly evaluate the amount of the microtubules present in the oocyte by measuring the retardance.

Compared to immunostaining or other microscopy methods, the Polscope® offers the advantage of being non-invasive to preserving oocyte viability while allowing repeated observations over time [31]. In the literature there is a general agreement about correlation between the presence of the spindle and fertilization rate during IVF [32–34] or embryo development potential [35]. This system has also been applied to evaluate spindle recovery after human oocyte cryopreservation using different protocols.

Rienzi *et al.* used a 1.5 M PROH plus 0.1 M sucrose slow-freezing protocol and showed a spindle recovery in 37% of the oocytes immediately after thawing [36]. In the subsequent steps, the spindles disappeared in all the eggs, only to reappear in all surviving oocytes within 3 h of incubation at 37°C. Similar data were published by Bianchi *et al.* using a comparable freezing protocol with higher sucrose concentration (0.3 M) [37]. Immediately after thawing, only 22.9% of oocytes showed a weak birefringence signal, while only 1.2% of oocytes displayed a high signal. Three hours after thawing, the proportion of oocytes exhibiting a weak or high intensity signal was 49.4 and 18.1%, respectively. Finally, after culture for 5 h following thawing, a weak birefringence signal was detected in 51.8% of oocytes, while 24.1% showed a high signal. There was a statistically significant increase in signal restoration after 3 h of culture ($P < 0.001$). Those data were partially confirmed later by confocal microscopy where unfrozen control oocytes were compared with frozen oocytes fixed at 0, 1, 2 and 3 h after thawing [38]. All the control oocytes (100.0%) displayed bipolar spindles with constriction evident at both poles. Following cryopreservation, there was a significant reduction of oocytes with bipolar spindles directly following thawing (T0; 59.1% bipolar), although after 1 h of culture (T1) 85.7% of oocytes regained bipolar spindles. Oocytes cultured for 2 (T2) or 3 h (T3) following thawing displayed 73.7 and 72.7% bipolar spindles, respectively. Moreover, at T2, oocytes from older patients (>36 years) showed a significantly lower rate (11.1%) of chromosome alignment compared to younger patients (<35 years) where 70.0% of oocytes displayed a normal configuration ($P < 0.05$). A similar trend was evident at T3 where only 41.7% of oocytes from older patients maintain chromosome alignment compared with 60.0% in oocytes from younger patients.

Confocal microscopy analysis has been used to generate more data from oocytes frozen using different protocols based on PROH and sucrose cryoprotectants at different concentrations (0.1 and 0.3 M) [40]. A higher sucrose concentration is responsible for a significantly improved survival rate, but this also might be associated with an increased osmotic stress for the oocyte [18] and consequent spindle damage. The confocal analysis on fresh and frozen thawed oocytes better clarified this hypothesis. The authors showed that, of the 104 oocytes included in the unfrozen group, 76 (73.1%) displayed normal bipolar spindles with equatorially aligned chromosomes. Spindle and chromatin organizations were significantly affected (50.8%) after cryopreservation involving lower sucrose concentration (61 oocytes), whereas these parameters were unchanged (69.7%) using 0.3 mol/l sucrose (152 oocytes). From those data it is possible to affirm that cryopreservation procedures induce damage in oocytes but that this is relatively acceptable using higher sucrose concentration. This can be explained by the higher dehydration reached with a 0.3 mol/l sucrose protocol that limits the amount of intracellular water and prevents ice crystal formation. Consequently, the spindle is better preserved. This is supported by the clinical data obtained using these protocols [41–43]. Additional ultrastructure investigations were performed comparing PROH with ethylene glycol (EG) as the permeating cryoprotectant. The frequencies of normal spindle configuration were lower

in frozen EG or PROH oocytes compared with fresh oocytes (53.8, 50.9 and 66.7%, respectively, $P < 0.05$) [44].

Besides the meiotic spindle, other subcellular structures have to be preserved during freezing–thawing procedures. The zona pellucida, a multilaminar structure composed of glycoprotein, has a fundamental role in the normal fertilization process. The sperm can bind to specific sites inducing the release of cortical granules that prevent the entrance of other sperm through glycoprotein inactivation [45]. During freeze–thaw procedures this structure can be damaged, thus compromising the post-thaw fertilization potential. The so called "zona hardening" was first described in mouse oocytes showing that the fertilization rate dramatically decreased after thawing.

Normally, the entrance of the sperm inside the egg causes a transient increase in the intracellular calcium concentration [46], causing release of the cortical granules and consequent sperm binding glycoprotein inactivation. This might be related to the use of penetrating cryoprotectant in the cryopreservation protocols that induce premature cortical granules release and is responsible for calcium oscillations inside the cell [47]. The entrance of the cryoprotectant is probably associated with a calcium flow toward the cell that causes an increase in the intracellular calcium concentration [48]. Moreover, it has been demonstrated that DMSO and PROH can also cause a proteolytic modification of the sperm binding protein ZP2. Adding fetal bovine serum to the freezing mixture can reduce the zona hardening and the inactivation of the ZP2 even though it does not prevent cortical granule release. A protein known as fetuin competes with the enzymes released from the granules which are responsible for the zona hardening [49]. In the human there is limited data on the fertilization rate by conventional IVF after thawing since the use of intracytoplasmic sperm injection (ICSI) technique could bypass the issues related to zona hardening. More data are available on the cortical granules loss. In 1988 Sathananthan *et al.* observed a reduction in numbers [50] that has been confirmed more recently using electron microscopy [44, 51, 52]. Ghetler *et al.* analyzed cortical granule exocytosis by either confocal microscopy or transmission electron microscopy (TEM) [52]. Mature oocytes exhibited increased fluorescence after cryopreservation, indicating the release of the granules; this was confirmed by TEM that revealed a drastic reduction in their amount at the cortex of frozen–thawed MII oocytes. They demonstrated that the use of PROH-freezing protocol for human oocytes resulted in extensive cortical granule exocitosis. Those results were later confirmed by Nottola *et al.* who showed that the amount and density of cortical granules appeared to be abnormally reduced in some frozen–thawed samples, despite the slow-freezing protocol [51]. These abnormal features were frequently associated with an increased density of the filamentous texture related to the occurrence of zona "hardening." The same result was obtained by these authors using EG and sucrose as cryoprotectants [44]. As previously mentioned, the MII oocyte has a complex subcellular structure that includes the meiotic spindle, cortical granules and other features like mithocondria or smooth endoplasmatic reticulum (SER). These ultrastructural components were not taken into account in the initial studies, since oocyte survival after thawing was primarily correlated to morphological appearance of the egg. Lately, several authors focused their attention on the possible consequences related to subcellular injuries. Nottola *et al.* analyzed fresh and frozen–thawed oocytes using a slow cooling method with PROH and sucrose (0.1 or 0.3 mol/l) as cryoprotectants [51]. The oocytes were then processed for electron microscopy observations. All the oocytes showed a homogeneous cytoplasm and an intact zona pellucida (ZP) with abundant and uniformly dispersed organelles (mainly mitochondria–smooth endoplasmic reticulum aggregates and mitochondria–vesicle complexes). Nevertheless, a degree of microvacuolization was detected in the ooplasm of some frozen–thawed oocytes, particularly in those treated with higher sucrose concentration. Another interesting study comparing fresh and frozen thawed eggs (PROH plus 0.3 mol/l sucrose) was published by Gualtieri *et al.* [53]. The authors showed evidence that in fresh samples mitochondria had a regular shape with few short cristae, whereas in the frozen–thawed group a high percentage of oocytes (72%) showed a variable and, in some cases, a very high fraction of mitochondria with decreased electron density of the matrix or ruptures of the outer and inner membranes. Moreover in those oocytes, the mitochondrial damage was associated with SER swelling.

## Slow cooling protocols and outcomes

As implied by the name, this protocol is characterized by a slow decreasing temperature rate. Several mathematical models have been used to define an optimal

curve applicable to oocytes since the freezing rate is vital to achieve sufficient and progressive dehydration, and thereby minimize the potential of intracellular ice formation.

The slow cryopreservation protocol applied worldwide to oocytes is the same as that normally used for embryo freezing, and it is based on a slow freeze–rapid thaw program. The cryoprotectant agents most frequently used are PROH and sucrose; the combination of penetrating and non-penetrating agents allow a better outcome in terms of oocyte stability. The oocytes are maintained for 10 min in a 1.5 M PROH solution with 20% protein supplement for equilibration phase. During this time the PROH enters the cell as water exits the oocyte. The second step is performed in a 1.5 M PROH solution supplemented with various concentrations of sucrose (according to different protocols) and 20% protein source. During this 5 min exposure, the oocyte increases its dehydration in a proportional way to the amount of sucrose contained in the solution. The oocytes are subsequently loaded in straws and placed in an automated Kryo 10 series biological vertical freezer (Planer Kryo GB). Freezing solutions are cooled from 20°C to –7°C at a rate of 2°C/min. Manual seeding of oocytes within straws is performed at near –7°C and this temperature is maintained for 10 min in order to allow uniform ice propagation. The temperature is then decreased to –30°C at a rate of 0.3°C/min and then rapidly lowered to –150°C at a rate of 50°C/min. Straws are then directly plunged into liquid nitrogen at –196°C and stored.

Thawing consists of rapid rewarming (air for 30 s and then 40 s in a 30°C water bath) and subsequent stepwise dilution of the cryoprotectants; first in 1.0 M and then in 0.5 M PROH solutions supplemented with sucrose (depending on the sucrose concentration used during freezing procedure) for 5 min each, and then in a sucrose solution for 10 min and in PBS solution for an additional 10 min. Finally, the oocytes are returned to culture media at 37°C to support recovery.

The first pregnancy using oocyte cryopreservation was obtained in 1986 with a protocol tested on mouse eggs [1]. The author used DMSO as permeating cryoprotectant and partially removed cumulus cells before freezing. The results were not the same as those obtained in the animal model; the main problem emerged following the first attempt which showed a low oocyte survival rate that was totally incomparable to embryo freezing–thawing results. Consequentially, this procedure was abandoned for many years. However, in the last 10 years, several prompted renewed interest; higher survival rates in cancer patients or the need to preserve fertility in general pushed several groups forward in improving this technique.

The first protocol used was the same Lassalle *et al.* applied to embryo freezing and was based on a 1.5 M PROH plus 0.1 M sucrose mixture [54]. Despite the great results on embryo survival, this protocol was not the one tailored for egg freezing. One of the first studies with this protocol was published in 2004 where, out of 737 oocytes the survival rate was just 37%, while the fertilization and cleavage rates were 45.4 and 86.3%, respectively [43]. The ICSI technique was used to inseminate after the first report of Porcu *et al.* but, even so, the rate of zygotes obtained was not very satisfying [55]. A total of 15 clinical pregnancies were achieved (25.4% per transfer and 22% per patient) with an implantation rate of 16.4%. The outcome was much lower in terms of success than results obtained with frozen embryos and analogous conclusions were reached by other groups using the same protocol.

The need to improve several aspects of oocyte cryopreservation procedures led to a wide set of experiments that allowed this technique to become a routinely used approach in IVF. Paynter *et al.* pointed out first the need to increase oocyte dehydration in order to raise survival rate [56]. The protocol suggested by Lassalle did not guarantee a sufficient water flow out of the cell thus causing intracellular ice formation and subsequent cell death [54]. The importance of a non-permeating agent (such as sucrose) in the freezing solution has been deeply analyzed; this, in fact, enhances oocyte dehydration. The higher the sucrose concentration, the more the cell dehydrates, as water rapidly leaves the cytoplasm to dilute the high concentration of extracellular solutes. The exposure time to cryoprotectant should be long enough to enable sufficient cell dehydration, but not so excessive as to damage the cell by altering the intracellular pH.

To this purpose, Fabbri *et al.* raised the sucrose concentration from the standard 0.1 to 0.2 and 0.3 M, improving the survival rates significantly (34, 60 and 82%, respectively) as a result of a more adequate dehydration [40]. The data were confirmed by several authors, but the worldwide results were often related to a small number of patients with restricted indications and low numbers of eggs [57–59].

The most exhaustive data were produced by Italian groups in the next few years due to introduction of the

Italian law (40/2004) in which oocyte freezing was no longer an option but rather a main pillar in the IVF routine.

During fresh cycles only a few oocytes can be inseminated; therefore, cryopreservation is the only option to avoid wastage of surplus eggs and consequent repeated ovarian stimulation. During the thawing cycles it is possible to use just a limited number of oocytes, which can lead to obvious disadvantages such as the poor or insufficient number of embryos transferred and consequent unsatisfactory pregnancy outcomes. This situation brought about a new challenge: the need to find a protocol that could maintain high survival and fertilization rates and enhance the implantation rate/oocytes. In this way, even the thawing of few eggs/cycles can lead to a high pregnancy rate. The first reports were mainly based on a slow freezing using high sucrose concentration (0.3 M); Chamayou *et al.* showed no difference in fertilization rate between fresh and sibling frozen–thawed oocytes cryopreserved with 0.3 M sucrose protocol [60]. The cleavage rate and embryo quality were significantly reduced in the frozen–thawed group ($P < 0.001$) confirming, once again, that an excess of dehydration may compromise the oocyte developmental potential. La Sala *et al.* despite the very good data related to fertilization, cleavage and embryo quality did not show an improvement in pregnancy and implantation rate (4.2 and 5.8%, respectively) [61]. The same result was obtained by Borini *et al.* on 927 oocytes from 146 patients [41]. Again, very good survival (74.1%), fertilization (76%) and cleavage (90.2%) rates were not supported by corresponding results in terms of pregnancy and implantation rates, which were still disappointing (12.3 and 5.2%, respectively). Another study was performed by Levi Setti *et al.* with analogous results [42]. Oktay in 2006 published a meta-analysis calculating the combined outcomes of 26 reports using slow freezing, mature oocytes and ICSI, published prior to June 2005 [62]. These studies included approximately 4000 thawed oocytes. Clinical pregnancy per thawed oocyte was 2.4%, while the implantation rate per transferred embryo was 13.1%. Analysis of the 7 studies using slow freezing published between June 2005 and March 2006 evidenced a clinical pregnancy per thawed oocyte ($n = 2409$) of around 2.2%, while implantation rate per transferred embryo was down to 6.5%.

More interesting was the retrospective study conducted by De Santis *et al.* who highlighted the differences between the two protocols applied in the literature at that time (using 0.1 and 0.3 M sucrose) [63]. It was surprising that, while the survival and fertilization rates were significantly improved using higher sucrose concentration, the pregnancy and implantation were much improved using low sugar-based solutions. This may be due to the subtle equilibrium between dehydration enhanced by sucrose and possible subcellular damages caused by the same cryoprotectant. At this point it was clear that, besides the "biological" good outcome, the high sucrose-based protocol was not producing good results in term of pregnancy and implantation.

Starting from this important observation, Bianchi *et al.* established a modified cryopreservation protocol in which the freezing solution contains 1.5 M PROH and 0.2 M sucrose in order to reduce the impact of shrinkage during cooling procedures [64]. The higher sucrose concentration (0.3 M) was used during thawing, after Fabbri *et al.* [40], in keeping with the original idea of Lassalle *et al.* [54], who used a thawing solution in which sucrose concentration was higher than that employed in the freezing solution. The survival rate reported was high and comparable to the previous studies (76.0%) as is the rate of fertilization (76.2%) and embryo cleavage (93.7%) [64]. The significant difference was evidenced in the pregnancy rate, which was 21.2, 18.9 and 21.8% per embryo transfer, thaw cycle and patient, respectively, and the implantation rate was 13.4%. Pregnancy rate per cryopreserved–thawed oocyte was 4.9% and the implantation rate per oocyte was 6.9%. These results represent an important achievement in the everyday application of oocyte freezing.

In a multicenter study, Borini *et al.* showed that, out of 2046 patients involved in oocyte cryopreservation, the overall survival rate of thawed oocytes was 55.8% [65]. In 940 thaw cycles, the mean numbers of inseminated oocytes and fertilization rates were significantly decreased versus fresh cycles outcomes ($2.6 \pm 0.7$ versus $2.9 \pm 0.2$ and 72.5 versus 78.3%, respectively), as were the rates of implantation (10.1 versus 15.4%), pregnancy rates per transfer (17.0 versus 27.9%) and pregnancy rates per cycle (13.7 versus 26.2%). Even though differences in clinical outcome were found among centers, a pregnancy rate per thawing cycle above 14% was achieved by most clinics.

It is evident that oocyte cryopreservation is able to add a valuable option to routine IVF procedures, especially in countries with severe law restrictions.

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Chapter 24

# Cryopreservation of human oocytes and the evolution of vitrification technology for this purpose

Michael J. Tucker and Juergen Liebermann

## Introduction

In principle, the aims we expect from a cryopreservation technique that allows us to store a biological material at the low temperature of liquid nitrogen (–196°C) with the tissue free of any crystalline structure, and with arrest of all molecular diffusion and chemical processes which would otherwise precipitate degradation and aging, are as follows:

1. To be able to arrest the metabolism reversibly.
2. To maintain structural and genetic integrity.
3. To achieve acceptable survival rates after warming.
4. To maintain developmental competence post-cryostorage.
5. For such a technique to be reliable, relatively simple and repeatable.

To accomplish these fundamental goals, two basic approaches have been developed: controlled slow-rate freezing [1] and a rapid cooling technique referred to as vitrification [2]. Specifically, the ability to be able to routinely cryopreserve human oocytes represents an important step forward in assisted reproductive technology (ART), and comes after more than two decades where studies of oocyte cryopreservation have long been the focus of unsuccessful efforts to perfect its clinical application. Very recently, however, vitrification as an alternative to traditional slow-freezing protocols has been shown to provide high degrees of success for cryopreservation of mature metaphase-II (MII) human oocytes.

In this chapter we shall consider the evolution of the practical application of cryopreservation technology to achieve consistently acceptable levels of cryosurvival of this highly cryosensitive gamete while retaining its inherent competency.

In general, the cryopreservation of any biological material includes six principal steps:

1. Initial exposure to a cryoprotectant, which promotes gradual cellular dehydration by partial removal of intracellular water.
2. Cooling (slow or rapid) to sub-zero temperatures (–196°C).
3. Secure storage at this low temperature.
4. Thawing or warming with gradual cellular rehydration.
5. Dilution and removal of the cryoprotectant agents and replacement of the cellular and intracellular fluid at a critical rate and temperature.
6. Ultimately, recovery and return to a physiological environment.

Compared to conventional slow-rate freezing where the concentration of the cryoprotectant is relatively low, and the cooling rate is very slow to avoid deleterious ice crystallization, vitrification is an ultra-rapid cooling technique that requires higher concentrations of cryoprotectant. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice crystallization (as in conventional freezing) but by extreme elevation in viscosity during cooling; in this approach the cells are placed into the cryoprotectant and then plunged directly into liquid nitrogen. Water is largely replaced by the cryoprotectant. For the vitrified state to be successfully achieved, cooling rates have to be high.

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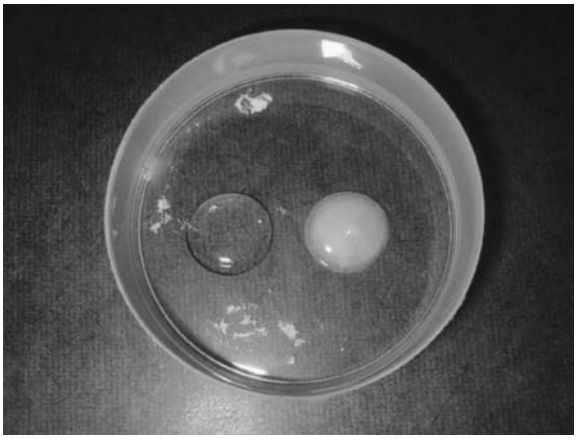

**Figure 24.1** Vitrification is the solidification of a solution ("glass formation") without ice crystallization. Two droplets of different solutions plunged directly into liquid nitrogen: the right droplet is pure Dulbecco's phosphate-buffered saline (DPBS) with ice crystallization; in contrast ,the left droplet contains an equi-molar combination of 15% ethylene glycol (EG) and dimethyl sulfoxide (DMSO) with 0.5 M sucrose in DPBS without ice crystallization (i.e. "glassification" in the vitrified state).

For example, with human oocytes the cooling rate has to be in excess of 15 000°C/min. During vitrification an aqueous solution is transformed directly from the liquid phase to a glassy vitrified state (Figure 24.1). With this method no ice crystals form that can damage the cells or the tissues. While the terms "freezing" and "thawing" are commonly used for conventional cryopreservation, the terms "vitrifying" and "warming" should be used for vitrification procedures. A particular problem for cells and tissues can be the high concentrations of cryoprotectants that are required to effect vitrification, as there exist biological limitations on the concentration of cryoprotectant that can be tolerated by the cells during vitrification. Thus, a major consideration during the development of vitrification protocols has been to attempt to increase the speed of temperature change, both during cooling and warming, while reducing the concentrations of cryoprotectants as low as possible without endangering the efficacy of the vitrification process [3].

In the clinical context there has been considerably less experience with vitrification technology in human ART, and it is still in the process of being accepted as a standard means to cryopreserve oocytes and embryos, although this situation is very rapidly changing both for cryostorage of embryos [4–16] and oocytes [17–28]. Indeed, the number of publications regarding vitrification in ART has increased exponentially over the last decade (Figure 24.2).

## Why cryopreserve human oocytes?

The last few years have seen a significant resurgence of interest in the potential benefits of human oocyte cryostorage [19, 29]. The benefits include the formation of donor "egg banks" to facilitate and lessen the cost of oocyte donation for women that are unable to produce their own oocytes, through greater ease of coordination and synchronization of donor and recipient cycles [30–32]. Also of major potential

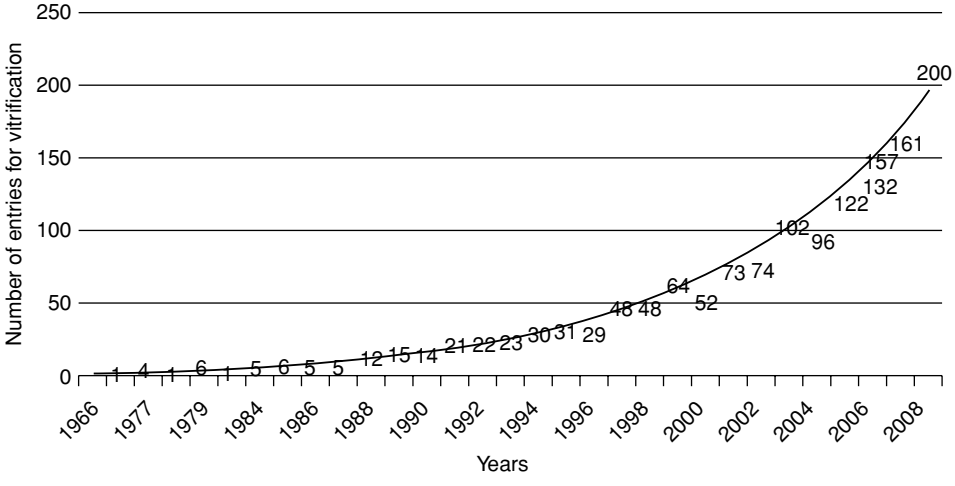


**Figure 24.2** The explosion of interest in the use of vitrification is beginning to affect clinical embryo and oocyte storage. (Data derived from the Scopus Library – the world's largest abstract and citation database of research literature and web sources; it offers access to 245 million references and 27 million abstracts from over 15 000 peer-reviewed journals.)

benefit is the provision of oocyte cryostorage services for women wishing to delay their reproductive choices for a variety of reasons. For women about to undergo therapy potentially harmful to their ovaries that may threaten their reproductive health, oocyte cryostorage represents the only truly proven clinical recourse [33]. Additionally, certain countries have restricted access to cryopreservation of human embryos by limiting the numbers of oocytes that may be inseminated; therefore, restricted insemination of oocytes has been undertaken with cryostorage of the surplus oocytes becoming the standard reproductive protocol, albeit with variable outcomes [20, 34]. Limited insemination of oocytes by patient choice (elective limited insemination [ELI]) probably has a burgeoning relevance for in vitro fertilization (IVF) worldwide as comfort grows with reduced superovulation, improved oocyte cryostorage and single embryo transfer. The growth in ELI will be driven both by women's appreciation of the legal benefits of "ownership" of their own gametes [29] and the improvement in IVF embryo selection technology [35]. There also exist certain clinical situations where oocyte cryopreservation can be applied to "rescue" an ovarian stimulation cycle; for example, in an IVF attempt where unexpectedly no spermatozoa are available, then the oocytes can be cryostored until such time as a resolution can be found, and this may even involve cryopreservation of both gametes and embryos [36]. Even with an intrauterine insemination cycle, where ovarian stimulation might be too exuberant, then oocyte retrieval and cryostorage can be applied, leaving one or two follicles intact to allow for intrauterine insemination (IUI) to be undertaken as planned to achieve live births [37] (M. J. Tucker *et al*., Unpublished data from Shady Grove Fertility RSC, 2007–9) (Table 24.1).

**Table 24.1** Clinical use of donor and autologous oocyte vitrification using the Cryolock (Biodiseño) carrier and M199-based vitrification media (Irvine Scientific, CA) or media as in Table 24.3

| | Donor oocytes | Autologous oocytes |
|---|---|---|
| Warming cycles | 12 | 21 |
| Oocyte survival (%) | 53/64 (83%) | 140/187 (75%) |
| Fertilization (%) | 48/53 (91%) | 101/140 (72%) |
| Transfers | 12 | 17 |
| Pregnancies/embryo transfer (%) | 9 (75%) | 5 (29%) |
| Implantations (%) | 9/24 (37.5%) | 7/48 (14.5%) |

Unpublished data from Shady Grove Fertility RSC, 2007–9.

## Relevance of human oocyte cryopreservation

The majority of oocyte freezing so far applied clinically has been based directly on traditional human embryo cryopreservation protocols. Such protocols utilize a "slow-freeze/rapid-thaw" approach necessitating use of a programmable freezer, and these protocols have produced to date the majority of the approximately 900 offspring worldwide [26]. This is in comparison to $>$ 500 000 offspring born from mostly conventionally cryopreserved embryos. Fortunately to date, no significant increase in abnormalities has been reported from these cryostored oocyte pregnancies [38], regardless of the historical concerns that cryopreservation of mature oocytes might disrupt the meiotic spindle and thus increase the potential for aneuploidy in the embryos arising from such eggs. These concerns have mostly been allayed by publications that show no abnormal or stray chromosomes from previously frozen oocytes [39], and fluorescence *in situ* hybridization (FISH) comparison of embryos from fresh and thawed oocytes show no increase in anomalies [40]. There also appears to be adequate recovery of the meiotic spindle post-cryopreservation whether using conventional or vitrification technology [41–43]. With respect to cryostorage of donated oocytes, there have been a number of reports that have described success with this approach [23, 30, 32, 44, 45]. Indeed this approach has now been formally commercialized.

Cryostorage of women's own oocytes was originally reported with three pregnancies established in the late 1980s by two centers [46, 47]. Following a hiatus of several years, these early reports were reproduced by others [17, 30, 48]. These pregnancies mostly arose from the freezing of oocytes that had been collected for purposes of infertility therapy where couples may have had religious or ethical concerns with embryo cryopreservation; or when couples consented to research studies; or even when spermatozoa were unexpectedly unavailable after oocytes have been retrieved during an IVF cycle. The latter circumstances would have occurred following an unsuccessful testicular biopsy or when the partner was unable to produce a semen sample for a variety of unanticipated reasons.

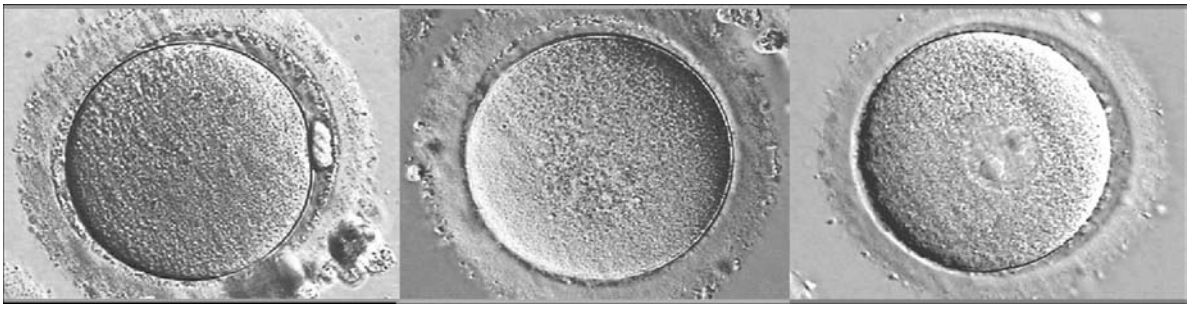

**Figure 24.3** (a) Mature metaphase-II (MII) stage oocyte; (b) immature MI stage oocyte; and (c) germinal vesicle (GV) stage oocyte.

All of these pregnancies arose from frozen–thawed mature oocytes, but for one notable exception, where a pregnancy arose from an immature germinal vesicle (GV) stage oocyte [49]. It has been considered that this stage of oocyte maturity might prove to be a more successful approach for cryopreservation because its oolemma is more permeable to cryoprotectant and its chromatin is more conveniently and safely packaged in the nucleus [50]. This has not proven to be the case, largely because such eggs still have to undergo GV breakdown and maturation to the MII stage before fertilization, and therefore their developmental competency is not so clearly established as with fully mature oocytes that are cryostored. The source of GV stage eggs, their specific derivation, and whether they have been exposed to any exogenous gonadotropins may well play a key role in the competency of such immature eggs [51]. Recently, with the increased interest with in vitro maturation (IVM) as an alternative to standard IVF with superovulation, oocyte cryopreservation has played an increasing role, although to date only mature oocytes have been successfully cryostored using vitrification after IVM [52].

Whether using mature oocytes or not, conventional freezing technologies have suffered from inconsistency in terms of cryosurvival. Consequently, there have been attempts recently to introduce significant modifications to overcome this apparent drawback. One approach has been to replace sodium as the principal cation in the cryoprotectant with choline that does not diffuse through the plasmalemma, in an attempt to reduce the potential for cell toxicity arising from high solute concentrations ("solution effects") that occur during cooling as the water becomes ice, so removing water from the system [53]. This cationic replacement has been shown to be beneficial in a mouse model of conventional freezing, but begs the question why embryos can routinely be safely and successfully frozen in sodium-based media [54]. Another modification that has been adopted has been to increase the concentration of the non-permeating cryoprotectant sucrose in the freezing media [55]. Such modifications appear to have improved clinical outcomes; indeed, higher sucrose concentration may improve cryosurvival. However, it may not necessarily improve overall clinical outcomes [56]. Intracytoplasmic sperm injection (ICSI) has become the accepted norm for insemination of oocytes post-thaw, to avoid any reduction in sperm penetration of the zona pellucida due to premature cortical granule release precipitated by the cryopreservation process [39, 57]. However, it has recently been suggested that the use of calcium-free cryoprotectant during vitrification of mouse oocytes may lessen zona hardening, thus increasing the fertilization of the oocytes following conventional insemination [58]. In any event, as cryostored oocytes after thawing/warming are often in low numbers and represent "precious" gametes, it seems only reasonable to apply ICSI, as an acknowledged successful adjunct to IVF technology, to achieve the highest consistent levels of fertilization.

Oocytes retrieved after controlled ovarian hyperstimulation will be principally mature (metaphase of meiosis II with extruded first polar body [MII]: Figure 24.3a). Oocytes from unstimulated cycles or following minimal use of gonadotropins may be mostly immature (intermediate post-germinal vesicle breakdown with no polar body extruded [GVBD] or MI stage and germinal vesicle stage [GV]: Figure 24.3 b, c). Oocytes may be cryopreserved at any stage, but mature or GV stage oocytes are preferred. If GV stage oocytes are cryostored, IVM of these eggs post-thawing will need to be accomplished before fertilization can be

attempted. Alternatively, immature oocytes may be left to mature prior to cryopreservation. A pregnancy from the former approach cryopreserving GV stage oocytes with subsequent thaw and IVM resulted in a successful live birth over 10 years ago [49]; however, the latter approach has proven to be more effective, with vitrification of only those oocytes that reach maturity in vitro [38, 59].

It has long been understood that unfertilized oocytes are more difficult to cryopreserve than cleavage stage embryos. This has largely to do with the oocyte's surface to volume ratio, the reduced membrane permeability of its oolemma, the temperature-sensitive metaphase spindle and zona pellucida and its susceptibility to parthenogenetic activation and chill injury. Membrane characteristics also vary between oocytes and embryos and between oocytes of differing maturities [60].

## Outcomes with oocyte cryopreservation

The scientific literature on oocyte cryopreservation seems to grow daily. Most reports focus on clinical pregnancy rates [61, 62] and, as such, while this data is helpful to increase our confidence with the technology, it does little to research new directions for oocyte cryopreservation. It can be difficult to establish baseline performance with any technology in human ART; however, an acceptable level of research can be undertaken in the area with appropriately donated tissue; i.e. with full Institutional Review Board approved patient consent. One such source is oocytes that remain unfertilized or unmatured the day after retrieval in IVF; or that are immature on the day of retrieval and not available as such for ICSI, which nevertheless reach maturity overnight and are donated by the woman undergoing IVF therapy. Table 24.2 is one such example that shows the oocyte post-warming survival rates after using two different carriers for vitrification: the Cryoloop and the Hemi-straw. A total of 928, aged and donated for research, human oocytes that failed to fertilize were subjected to vitrification. (The oocyte survival rate following 24 h of culture appeared lower in the Cryoloop group than in the Hemi-straw group [80.6 versus 85.4%], but this difference was not significant [$\chi^2$; $P = 0.061$].) Overall the percentage of surviving oocytes was 83.0% (771/928), which is a level that has helped promote clinical introduction of this technology for oocyte cryostorage (Table 24.2) [63].

**Table 24.2** Post-warming survival of human failed-fertilized oocytes after 24 h culture relative to the different vitrification carriers used

| | Vitrification technique | |
|---|---|---|
| | Cryoloop | Hemi-straw system |
| Number of oocytes | 448 | 480 |
| Oocyte survival | 361 (80.6%)[a] | 410 (85.4%)[b] |
| Overall survival | 738/890 (82.9%) | |

[a] From Liebermann and Tucker [63].
[b] $P = 0.061$, according to $\chi^2$ test.

## Improving current oocyte cryopreservation protocols

Oocyte cryopreservation is finally entering the mainstream of techniques in assisted reproduction. For sometime the reticence to apply oocyte cryopreservation was largely due to the perception that there were no obvious clinical niches requiring its routine use. As a reproductive technology it has more recently suffered from the stigma of being seen as "experimental" (ASRM Committee Opinion; http://www.asrm.org/Media/Practice/Essential_elements.pdf). This is changing due to legislation that has restricted use of embryo cryopreservation in some countries, and to a growing awareness on the part of certain infertile women that choose not to inseminate all of their oocytes either for ethical or practical issues (ELI). Cryostored oocytes are the "property" of only the woman, as opposed to cryostored embryos that are "co-owned" [29]. It was predicted over 10 years ago that the most likely area of early routine adoption of oocyte cryopreservation was to facilitate oocyte donation, as was illustrated at that time by the first offspring that arose from cryostored donor oocytes [30]. In any event, practice makes perfect is the key here, so that the more clinical experience we gain, the more we will hone our skills with oocyte cryopreservation. Animal models go only so far to enable us to improve human assisted reproduction; ultimately, it is clinical application which is the key to improvement, as has been seen with embryo cryopreservation. With oocyte donor "egg banking," information in terms of clinical success of protocols is generated within months not years, as would be the case with cryostorage of oocytes

**Table 24.3** Typical vitrification and warming media

| | EG (%) | Sucrose (M) | DMSO (%) |
|---|---|---|---|
| Equilibration solution | 7.5 | 0.0 | 7.5 |
| Vitrification solution | 15 | 0.5 | 15 |
| Warming solution | 0 | 1.0 | 0 |
| Dilution solution | 0 | 0.5 | 0 |
| Holding solution | 0 | 0.0 | 0 |

DMSO, dimethyl sulfoxide; EG, ethylene glycol.
Holding solution: hepes-buffered human tubal fluid (modified-HTF) plus 20% protein supplement (e.g. SPS; Sage Biopharma, CA).

for single women concerned with their future reproductive choices ("insurance freezing"/self donation) [64]. In accepting that human oocyte cryopreservation is here to stay, it is of great importance that we research the consequences of this technology carefully, to ensure that we do no harm, through establishment of an effective registry of outcomes and births from oocyte cryopreservation.

Conventional cryopreservation has seen significant improvement in consistency of outcomes the past few years in the form of the introduction of ICSI as the routine insemination approach after cryostorage, choline-based (sodium-depleted) cryo-media and use of higher concentration sugar solutions. Within the confines of conventional freezing technology there may be subtle variations in approach that will only be revealed through ongoing clinical studies. Major leaps of technology, such as injection of trehalose into the oocytes before freezing [65], while theoretically attractive, nevertheless impose an increased level of invasiveness that seems to run contrary to the wish to simplify oocyte freezing. However, we should remain open minded given that little has been done to date to assess oocyte quality at the cellular level, other than to determine the status of the meiotic spindle [41].

With regard to vitrification as a means to cryopreserve oocytes, increased speed of cooling through use of better designed carriers and protocols that lessen the concentration of cryoprotectant used, while hastening exposure and procedure times, have put this technology on the map [66], and excellent embryo quality can be obtained from vitrified oocytes [67]. Furthermore, the most widely used protocol for vitrification of biological material is a two-step partial equilibration in the cryoprotectant, and a three-step warming procedure in sucrose only (Table 24.3).

## Establishing and troubleshooting clinical oocyte cryostorage

In an attempt to help clinics to set up or evaluate oocyte cryopreservation as a standard clinical procedure, a series of considerations are made here to assist with focus on how to achieve consistent success:

- Finding the right carrier for loading and storing oocytes is essential. A wide variety of carriers is available, which can be either "open" or "closed/sealed" systems. An important requirement for a carrier is that it provides safety, easy handling, high recovery rate as well as high cooling rates (Figures 24.4 and 24.5). In addition, achieving consistent success with vitrification technology requires a distinct "learning curve" (e.g. Figure 24.6).
- Selection of oocytes for cryopreservation is critical for consistently good cryosurvival and acceptable clinical outcomes from use of such oocytes. Selection criteria, in addition to noting the precise maturational stage (see Figure 24.3a–c) include: size (to avoid use of oversized "giant" oocytes), morphology and cytoplasmic "health" (avoidance of vacuolation, heterogeneity, etc: Figure 24.7).
- If, in spite of good selection practices for the oocytes to be cryopreserved, cryosurvival is poor, then the following potential problem areas should be investigated:

  1. Test the cryoprotectant media, vitrification carrier plus other consumables and the protocol in general with a mouse oocyte bioassay.
  2. Confirm the appropriate temperatures for work surfaces pre-cryopreservation and postcryopreservation.
  3. Confirm that production or delivery of the cryoprotectant media were undertaken correctly.
  4. With respect to vitrification in particular, be aware of how vitrification is less "protocol-driven" with less precise timing of steps, given that passage of the oocytes both into and out of the vitrification and warming solutions is more dependent on what is actually happening to the cell in terms of shrinkage and re-expansion (Figures 24.8–24.11). Thus, a flexible approach is highly recommended.

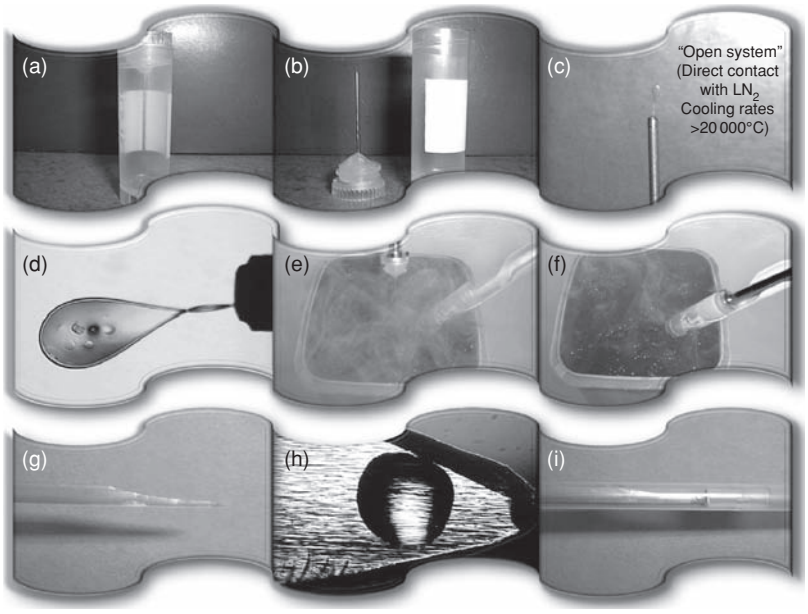


**Figure 24.4** Vitrification carriers. (a–f) Cryoloop; (g–i) Hemi-straw ("homemade").

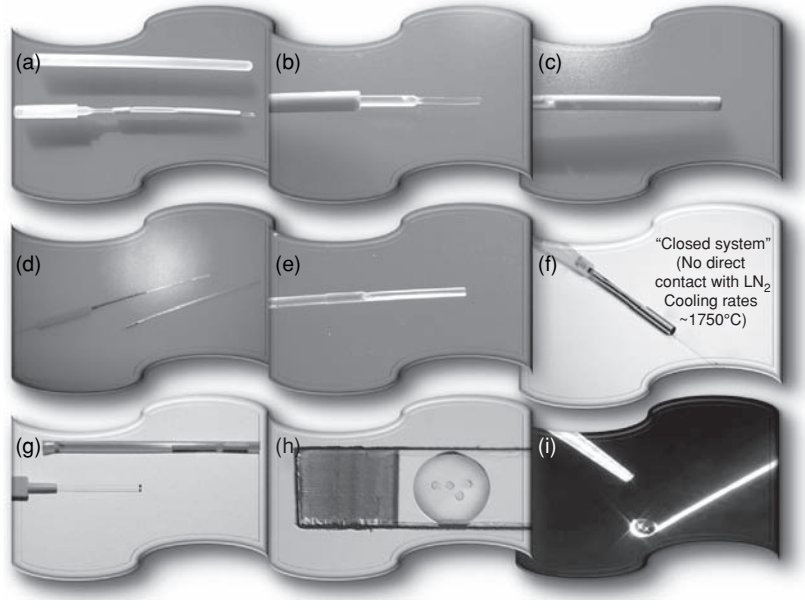


**Figure 24.5** Vitrification carriers.(a–c) Cryoleaf; (d–e) HSV (High Security Vitrification Kit); (f) Cryotip; (g–h) Cryotop; (i) Fibre Plug.

5. Finally the speed of both the final vitrification step and the warming step out of the liquid nitrogen is critical, such that if these steps are delayed, cryosurvival may well be compromised due to an increased potential for ice crystal formation.

Note that morphological cryosurvival itself is not the sole criterion to judge the success of oocyte cryopreservation: the oocytes themselves must be able to fertilize normally at an acceptable rate; develop morphologically as would fertilized fresh oocytes; and ultimately to implant at a clinically appropriate rate. Poor embryonic cleavage is especially indicative of cryptic cyto-skeletal damage that may have incurred during the cryopreservation process. It has to be remembered, however, that oocytes are only as good as they were

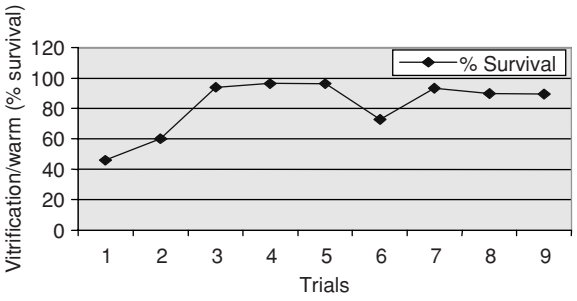


**Figure 24.6** Oocyte vitrification preclinical studies at Reproductive Specialists of New York for one embryologist using a Cook Flexipet tip within a sealed Cryo-straw where a distinct "learning curve" occurred. Cumulative viability post-warming from all trials was 144/168 (85.7%), establishing clinical acceptability for that individual.

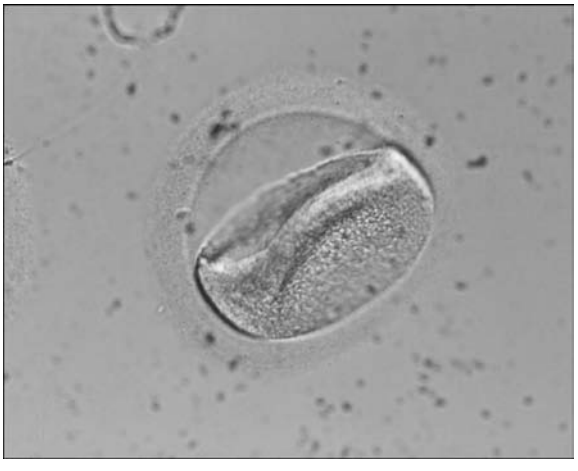

**Figure 24.8** Collapsed metaphase-I (MI) oocyte in 7.5% ethylene glycol (EG)/7.5% dimethyl sulfoxide (DMSO) prior to vitrification.

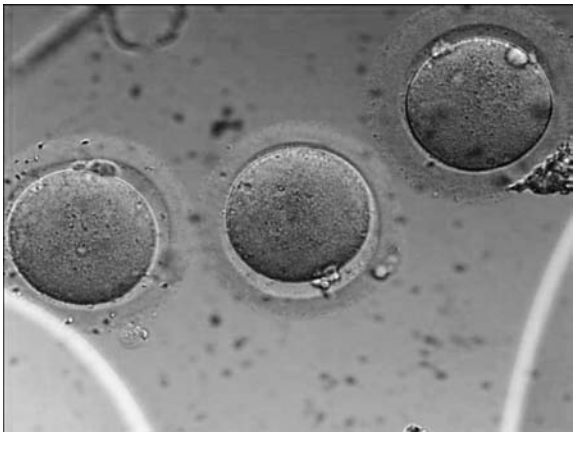

**Figure 24.10** Mature metaphase-II (MII) oocytes post-vitrification.

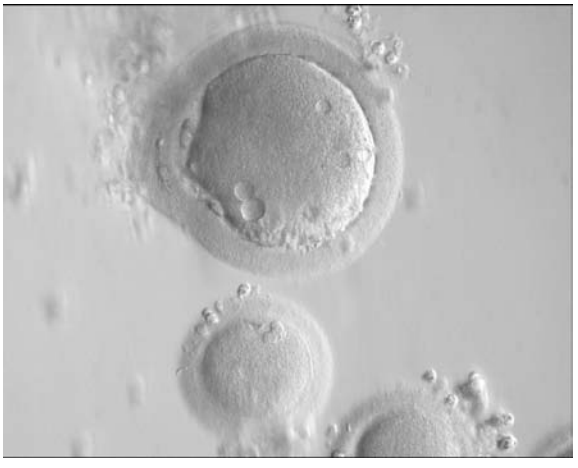

**Figure 24.7** Preselection of oocytes for vitrification. For example, an oversize (probably diploid), dysmorphic and vacuolated oocyte after "stripping" in hyaluronidase (40 IU/ml).

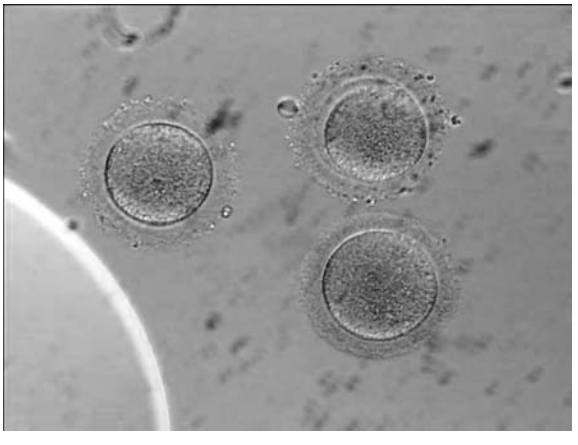

**Figure 24.9** Immature metaphase-I (MI) oocytes post-vitrification (note no polar body present).

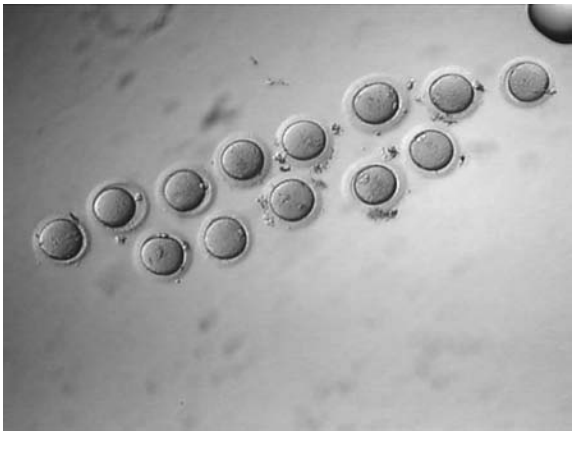

**Figure 24.11** Human oocytes after vitrification in 15% ethylene glycol (EG)/15% dimethyl sulfoxide (DMSO) plus 0.5 M sucrose, with warming in dilutions of sucrose.

at their time of collection, and fundamental compromise of oocyte quality may occur during ovarian stimulation, retrieval and, more problematically, innate quality issues related to a woman's physiology may make assessing these developmental issues less clear cut. Indeed, there exists a natural variability between women, and often between stimulation cycles from the same woman; such fluctuations in oocyte quality may have a significant negative impact on innate competency of such eggs. However, it is our mission during oocyte cryopreservation to minimize any deleterious conditions that reduce oocyte quality further on a permanent basis.

## The continuing evolution of oocyte cryopreservation

Before the currently established vitrification protocol becomes "fossilized" as the standard approach for oocyte cryostorage, it is important to consider the effort and studies that have been undertaken over the years to achieve success with this technique. So it remains important for us to continue to seek improvement in outcomes for all women regardless of their needs for oocyte cryostorage and the quality of their oocytes. There are two main ways to efficiently achieve the vitrification of water inside cells. One is to increase the temperature difference between the samples and vitrification medium, and the second is to find materials with fast heat conduction. However, the actual rate during vitrification procedures may vary extremely depending on the device used, technical proficiency and even the specific movement of the carrier at immersion into the liquid nitrogen. Every oocyte has its own optimal cooling rate, and oocytes are cells that are exquisitely prone to chilling injury. To date vitrification as a cryopreservation method has had relatively little practical impact on human assisted reproduction. With improving awareness of the technology, plus its refinement especially in the commercial arena, added to the growing numbers of reports of successfully completed pregnancies following vitrification makes for an extremely encouraging future for its wider adoption, and it bodes well for the wider spread use of vitrification for cryopreservation of both human oocytes and embryos. Whether there exists a need to improve cooling rates yet further with use of low-pressure "slush" liquid nitrogen machines [68], use of high hydrostatic pressure systems to "condition" oocytes prior to vitrification [69] or to undertake cooling on supercooled metal surfaces [70] remains to be seen. Such developments do detract from the simplicity and low-cost benefits of vitrification; however, even higher cooling rates may reduce even further the need for the relatively high concentrations of cryoprotectant and so allow vitrification to be an even more low-impact cryopreservation approach for oocytes.

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Chapter 25

# Cryopreservation and transplantation of isolated follicles

Marie-Madeleine Dolmans and Anne Van Langendonckt

## Concept of minimal residual disease

For pre-pubertal patients and women who cannot delay the start of chemotherapy, cryopreservation of ovarian tissue is the main option available to preserve their fertility before cancer treatment. Indeed, the prospect of reversing treatment-related premature ovarian failure by autotransplantation of frozen–thawed ovarian tissue harvested before chemoradiotherapy is becoming increasingly real, with 11 live births already reported using this technique [1–7]. It is expected that, in the near future, an increasing number of cancer patients cured of their disease will request re-implantation of cryopreserved ovarian tissue [8].

However, very little has been published on the safety of ovarian tissue transplantation in cancer patients [9]. One major concern raised by the use of ovarian cortical strips is the potential risk that the frozen–thawed ovarian cortex might harbor malignant cells. This could induce a recurrence of the disease after re-implantation if biopsy is carried out before chemotherapy. Shaw *et al.* previously reported that ovarian grafts from AKR mice could transfer lymphoma to recipient animals [10]. More recent studies tested the safety of cryopreserved human ovarian tissue from lymphoma patients for transplantation, and suggested that ovarian tissue transplantation in Hodgkin's disease is safe [11–13]. Selection of patients for the procedure should therefore take into account the malignancy type and its activity. In breast cancer, for example, metastasis may spread to the ovaries [11], though a recent study by Sánchez-Serrano *et al.* did not evidence malignant cells in ovarian cortex from breast cancer patients by immunohistochemistry [14]. In leukemia, malignant cells may be present in the bloodstream, with the risk of transferring leukemic cells [15].

Hematological malignancies are the most frequent indication for ovarian tissue cryopreservation in our department, and represent 44% of all malignant indications. Among them, Hodgkin's lymphoma accounts for 22% of cases, followed by leukemia (11.3%) and non-Hodgkin's lymphoma (11%) (Figure 25.1).

Because frozen–thawed ovarian tissue may contain malignant cells that could potentially lead to disease recurrence after re-implantation, we decided to conduct a study to evaluate the presence of leukemic cells in cryopreserved human ovarian tissue from patients with chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). Detection of minimal residual disease in ovarian tissue was carried out by histology, real-time quantitative polymerase chain reaction (RT-qPCR) and long-term xenografting (6 months).

Histology did not reveal any malignant cells. By RT-qPCR, 2 of the 6 CML patients were positive for BCR-ABL in their ovarian tissue, while among the 12 ALL patients, 7 of the 10 with available molecular markers showed positive leukemic markers (translocations or rearrangement genes). Four mice grafted with ovarian tissue from ALL patients developed intraperitoneal leukemic masses.

In conclusion, this study evidences, by RT-qPCR, ovarian contamination by malignant cells in acute as well as chronic leukemia, while histology fails to do so. Moreover, our results also indicate that chemotherapy before ovarian cryopreservation does not exclude malignant contamination. Finally, re-implantation of

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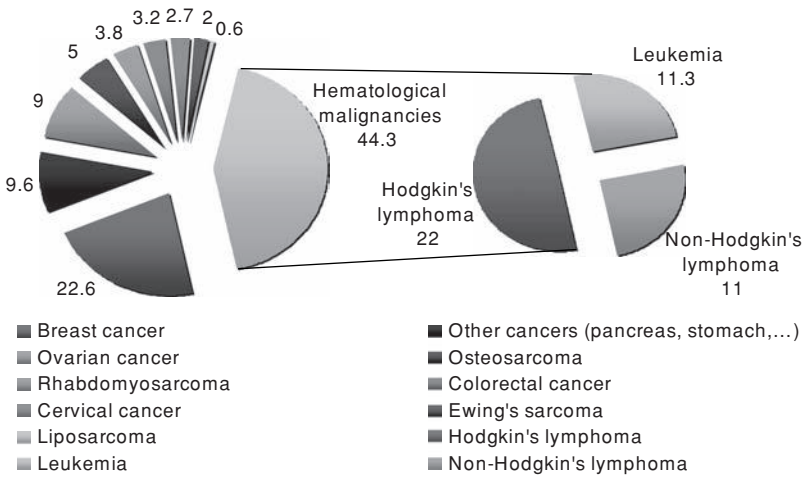


**Figure 25.1** Indications for ovarian tissue cryopreservation in case of malignant disease at Saint Luc's University Hospital, Brussels, Belgium in 2008. See plate section for color version.

cryopreserved ovarian tissue from leukemic patients puts them at risk of disease recurrence.

## Concept of follicle isolation

To avoid transferring malignant cells together with grafted ovarian tissue, ovarian follicles may be isolated from the surrounding tissue and then either cultured for in vitro follicle maturation or transplanted directly into the recipient.

Culturing isolated follicles from the primordial stage is a particularly attractive proposition, since they represent more than 90% of the total follicular reserve and show high cryotolerance [16]. It is widely reported that isolated primordial follicles do not grow properly in culture [17, 18], because follicle isolation [18, 19] or partial follicle isolation[17, 18] severely impairs follicular viability in culture, and that primordial follicles develop best in organ culture. Indeed, encouraging results were achieved by Hovatta's team [20] and Telfer's team [21] where follicles grown in culture of human ovarian cortical strips could be isolated at the secondary stage and grown in vitro to the late pre-antral/early antral stage. Nevertheless, recent publications show that encapsulation of isolated pre-antral follicles in alginate beads may be a suitable system for in vitro culture of small human pre-antral follicles, as they can survive and grow after enzymatic isolation and in vitro culture for 7 days [22]. However, this in vitro approach is difficult in humans due to the prolonged duration of folliculogenesis, and obtaining antral follicles from primordial follicles in vitro remains challenging.

This led us to consider an alternative strategy, which involves grafting of isolated ovarian follicles. For human primordial follicles, mechanical isolation is not possible due to their size (30–40 μm) and their fibrous and dense ovarian stroma, so enzymatic tissue digestion with collagenase is generally used. Different types of collagenase (Ia, II, IX, XI) have been employed for this purpose, either alone [17, 23] or in combination with DNase or mechanical isolation [18, 19, 24–28]. However, the drawback of collagenase, which is a crude preparation derived from *Clostridium histolyticum*, is that it may contain high endotoxin levels that could severely impair culture and grafting outcomes. It also shows substantial variations in effectiveness between batches [29], which may explain the discrepancies in results between different groups. This appears to indicate that some crude collagenase preparations may contain components interfering with follicle quality.

The inconsistent results obtained with collagenase thus prompted us to set up a new follicle isolation protocol using Liberase. Indeed, in order to enhance the chances of follicular survival after isolation, enzymatic digestion procedures for human ovarian tissue needed to be optimized and standardized. Liberase is a blend of highly purified enzymes that has been successfully used to improve the quality of human pancreatic islet isolation [30].

We were able to obtain good quality isolated human follicles using the Liberase enzyme blend, showing good morphology and integrity and high viability (Figure 25.2). Morphology of the isolated follicles was assessed using DAPI staining with the

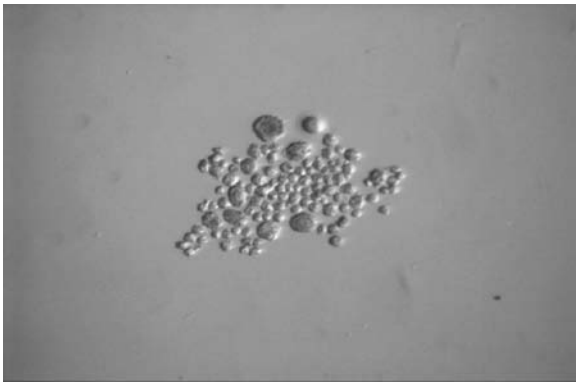

**Figure 25.2** Enzymatically isolated human follicles seen under a stereomicroscope.

M1–M4 classification [31], based on granulosa cell layer integrity and oocyte extrusion. Viablity of the isolated follicles was assessed after exposure of the follicles to calcein and ethidium homodimer-I, while ultrastructural investigation by transmission electron microscopy provided us with additional qualitative information on follicle morphology after enzymatic isolation. Obtaining good quality isolated follicles is an absolute prerequisite for their further processing, either for culture or transplantation. This purified endotoxin-free enzyme preparation is a promising alternative for the reproducible isolation of intact primordial and primary follicles from human ovarian tissue. Moreover, with a view to clinical application and in accordance with new European laws, good manufacturing practice (GMP) blends are now available.

After ovarian tissue digestion by enzymes, follicles need to be recovered one by one with a glass micropipette under a stereomicroscope, which can be very exacting step if the follicle suspension contains a lot of isolated stromal cells and debris. This is why we developed a method for the recovery of isolated pre-antral follicles, based on the Ficoll discontinuous density gradient separation technique [32]. In the literature, filtration of the digested cortical cell suspension is widely employed to remove stromal debris and harvest follicles in different species. However, when this method was used in our laboratory, extensive follicular loss was noted on the mesh filter surface, with follicles remaining stuck to the filter after rinsing. The Ficoll discontinuous density gradient separation technique successfully addresses this problem and provides a suitable means of recovering large numbers of viable primordial follicles and separating them from the stromal cells. After investigating for follicles in the different interfaces, we found 63% in the phosphate-buffered saline (PBS)–1.06 Ficoll interface and 36.9% at the 1.06–1.09 Ficoll interface, which represents 99.9% of total recovered follicles. Analysis by vital fluorescent staining showed that 95.8% of follicles treated with Ficoll were totally viable. The Ficoll density gradient method thus allows us to maximize recovery of isolated human ovarian follicles and minimize manipulation time, while maintaining high follicular viability [32].

## Concept of follicle transplantation

Another approach to avoid transmission of malignant cells could be to transplant a suspension of isolated follicles. Transplantation of frozen–thawed isolated primordial follicles has been successfully carried out in mice [33], yielding normal offspring. As the follicular basal lamina encapsulating the membrana granulosa excludes capillaries, white blood cells and nerve processes from the granulosa compartment [34], grafting fully isolated follicles may be considered safe.

After isolation and recovery of human follicles, our aim was to evaluate the developmental capacity and viability of these isolated human follicles after transplantation. They were therefore embedded in plasma clots, serving as vehicles to facilitate subsequent grafting, and xenografted for 1 week to nude mice. Our study clearly showed that, after xenografting, isolated human primordial follicles were able to survive and grow [35]. Their survival and growth was evidenced by their morphologically normal structure and follicular stage at histology, as well as positive staining for Ki-67, a nuclear antigen associated with cell proliferation.

Another finding of the study was follicular activation 1 week after transplantation, as demonstrated by the decrease in the percentage of primordial follicles and increase in the percentage of primary follicles, accompanied by a much higher proportion of Ki-67-labeled follicles in the grafts. This phenomenon was observed after both cortical tissue and isolated follicle grafting, and may lead to premature depletion of the graft. Further studies are clearly required to better understand these events occurring during the first post-transplantation days, in order to preserve the ovarian reserve and prolong graft survival. A proper balance between inhibitory and stimulatory factors appears to be essential to control primordial follicle activation. Our hypothesis is that, after

grafting, this equilibrium might be disturbed due to a lack of growing follicles and disruption of the vascular supply. Previous studies, including those from our laboratory, have evidenced factors possibly involved in the control of the primordial to primary follicle transition [36, 37].

We then extended the grafting period to assess the developmental capacity of these enzymatically isolated human follicles after 5 months xenografting to severe combined immunodeficient (SCID) mice. The results showed that isolated human primordial follicles are able to survive after long-term xenografting, and can develop into antral follicles after follicle stimulating hormone (FSH) stimulation [38].

As this approach has successfully restored fertility to mice [33], our optimization of follicle isolation and recovery protocols now allows us to consider its development for humans.

## Concept of artificial ovary

Isolation of human follicles could be done directly after tissue harvesting on fresh ovarian tissue, as it has been done in experimental studies so far. These isolated follicles could then be cryopreserved and banked until use, as described previously with sheep follicles [39]. Although the easiest and most logical way to select is probably to cryopreserve the ovarian biopsy, in strips or fragments, and thaw them the day of isolation and grafting, it needs to be investigated whether there could be an advantage in cryopreserving follicles over tissue. The cryoprotectant permeation might be more efficacious through a cell suspension than through tissue blocks.

Reseeding isolated follicles, free of cancer cells, could be considered to restore fertility to patients where the risk of re-introducing malignant cells by ovarian tissue autografting cannot be excluded. This technique requires new surgical approaches to increase efficiency of graft recovery. Therefore, new artificial matrices might play an important role. Indeed, tissue engineering is an expanding field with more and more applications. New matrixes have been developed based on hydrogels, foams or natural polymers which could be suitable to nestle the isolated follicles and form a kind of artificial ovary before grafting to the patient. This would allow patients at risk of ovarian metastasis to benefit also from ovarian tissue cryopreservation and transplantation.

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Chapter 26

# ART and oocyte donation in cancer survivors

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## Introduction

This book is primarily about prevention; its emphasis is on interventions that can be done at the time of cancer diagnosis – modifications of treatment and techniques for storing gametes, tissues or embryos for future use. By contrast, this final chapter of Section 6 explores the options open to the cancer survivor some time after the diagnosis has been made and treatment completed. If preventive treatment was successful, in some cases, normal fertility has been preserved. Other survivors may conceive using the gametes, embryos or tissue that were obtained and cryopreserved before their gonadotoxic treatment.

However, in some cases, prevention may not have been successful. This chapter is written to provide insight into the fertility management of cancer survivors with compromised or absent ovarian function and without cryopreserved material.

Before embarking on a pregnancy, no matter how it is to be achieved, a cancer survivor and her physicians must look at her general health, at her uterine function, at her chance of recurrence and, finally, at her prognosis for long-term survival. Since these issues are common to a cancer survivor's pregnancies – whether achieved in the natural cycle, through assisted reproductive technology (ART) or through donor egg (DE) – they will be discussed together in the first part of this chapter.

The second part of this chapter will focus on situations where the cancer survivor does not conceive spontaneously but her fertility is amenable to treatment. In situations where ovarian function persists but is attenuated, ART may be able to overcome treatment-related damage. For some cancer survivors, specific modifications of ART may be indicated.

The third part of this chapter explores situations in which prevention has failed, was not possible or was not the right choice for a particular individual. We emphasize here that DE is not always the last resort. For a variety of different reasons that will be explored later, some women may choose DE as the best reproductive choice in a difficult situation.

## Evaluation of the cancer survivor for pregnancy

We begin, then, with the evaluation of the cancer survivor for pregnancy. A flow-chart of cancer survivor screening is shown in Figure 26.1.

### Prognosis

Before other evaluation is done, the treating oncologist should be consulted about the prospective mother's prognosis. Has the survivor been disease free for a sufficient period of time? Is her prognosis for cure generally good? Is there a risk that pregnancy would increase cancer recurrence? Kavic and Sauer have suggested that in some cases, but particularly in cases where cancer is considered to be in remission but not cured, a second opinion from an independent oncologist may be helpful [1]. Patients and their physicians may elect to proceed with pregnancy in the presence of a poor prognosis, or even in situations where pregnancy may increase the risk of recurrence. However, this should not take place without full discussion and fully informed consent from all parties involved.

### Chronic health problems

Additionally, 75% of survivors of childhood cancer will have at least one chronic health problem.

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
Published by Cambridge University Press. 

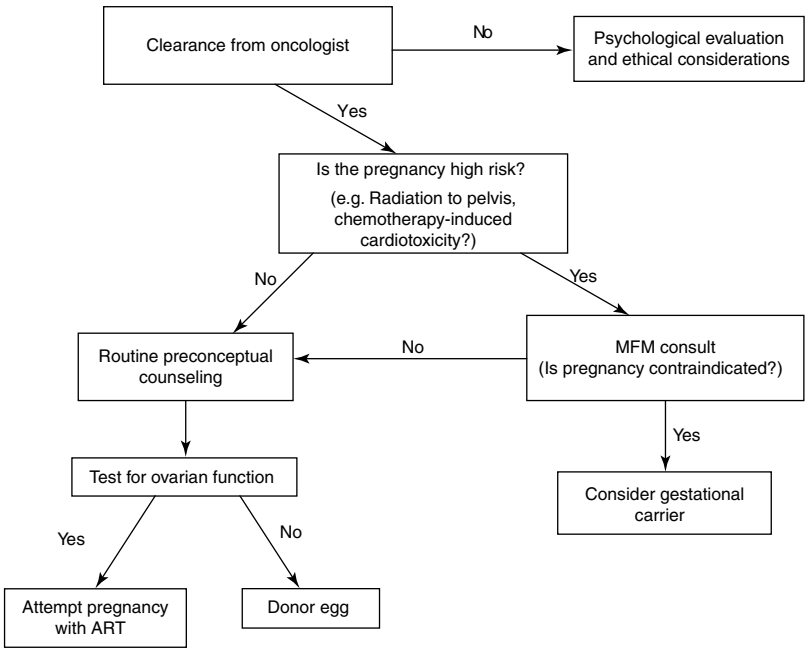


**Figure 26.1** Screening of the cancer survivor for pregnancy (spontaneous, assisted reproductive technology [ART] or oocyte donation). MFM, Maternal Fetal Medicine.

Cardiotoxicity from anthracyclines or mediastinal irradiation is common. Nephrotoxicity may result from ifosfamide and cis-platinum chemotherapy, radiotherapy, surgery or immunotherapy. Finally, 1 in 25 survivors of childhood cancers will develop a second primary cancer [2]. Thus, a cancer survivor contemplating parenthood should be evaluated for organ-system damage, and counseled about risks to herself and the fetus during pregnancy. When indicated, she should consult with the obstetrician who will care for her during pregnancy. She should also be made aware of any significant health risks that might affect her ability to raise a child to adulthood.

## Time interval between treatment and pregnancy

Depending on the nature of the cancer and the type of gonadotoxic treatment, oncologists may recommend that cancer survivors wait at least a year before attempting pregnancy [3]. The interval gives the oncologist time to evaluate for recurrence. Furthermore, it may minimize toxic effects of chemotherapy on the developing fetus. Experimental studies in animals have supported concerns about the risk to the fetus from exposure to chemotherapeutic agents. For example, mice that were exposed to chemotherapy 3 weeks prior to conception had high malformation rates and malformation rates remained 10-fold higher than the control group for conception up to 9 weeks after exposure [4]. In humans, pregnancy outcomes reported in women previously exposed to potentially mutagenic therapies are reassuring; however, these pregnancies often occur many years after exposure [5, 6]. A wait of at least a year is generally recommended; however, a safe time interval from chemotherapy to pregnancy has not been determined. Younger women with good ovarian reserve may wish to wait for longer.

Breast cancer survivors, by contrast, are usually advised to wait 2 years after completing chemotherapy before attempting pregnancy. Breast oncologists make this recommendation primarily because of the high rate of recurrence in the first years after diagnosis and not necessarily because of the effects of treatment on pregnancy outcome. The recommended time interval may be different depending on the age of the patient and stage of disease – with longer intervals recommended for younger patients and those with more advanced stage disease. Disease-free breast cancer survivors who conceive more than 2 years after finishing treatment do not appear to be at any increased risk of recurrence as compared to those who do not become pregnant following treatment [3].

This imposed delay causes concern for many women – they are aware of the higher risk of premature ovarian failure and the reduced chance for pregnancy as time passes. We can help to ease the anxiety by giving information and support and by not suggesting delay of treatment unless justified.

### Uterine evaluation

If a cancer survivor does not achieve a pregnancy in the first few months of attempting conception, she should generally have a uterine cavity evaluation – a hysterogram or sono-hysterogram – to rule out adhesions, intraluminal pathology or a significant septum. The uterine evaluation is especially critical for a woman who has had pelvic or total body irradiation.

Pelvic irradiation often results in damage to the uterine musculature and vasculature. Childhood radiation, especially, may result in poor uterine growth during puberty. The smaller uterine volume may diminish implantation, cause second trimester loss, preterm labor or placenta accretia [7]. A tripling of spontaneous miscarriage and a 10-fold increase in low-birth weight infants have been reported in patients who had received pelvic radiation therapy (RT) compared to the general population [8].

These risks are affected by the dose of RT delivered to the uterus and by the temporal association with puberty. There is, for example, a linear correlation between the size of the uterus, the response to hormone treatment and the age at which RT was administered [8]. Some investigators have emphasized that many reported pregnancies post-pelvic radiation are delivered prematurely and do not result in the delivery of a healthy child [9].

Although the adult uterus is less sensitive to the effects of radiation, adult cancer survivors who received RT to the pelvis should delay pregnancy for at least a year following RT; it has been suggested that pregnancies that occur <1 year after RT have a higher rate of low birth weights and miscarriages [10]. (For a more detailed discussion of the effects of pelvic radiation on uterine function, see Chapter 2.)

### Use of a gestational carrier

In individuals in whom a pregnancy is contraindicated, i.e. where carrying a pregnancy poses a significant risk to the mother or child, a gestational carrier may be considered. If ovarian function is sufficient and there is no medical contraindication to ovarian stimulation or retrieval, the patient's own oocytes can be retrieved and fertilized for transfer into a hormonally synchronized gestational carrier. In cases of low or absent ovarian reserve or where there are contraindications to ovarian stimulation, oocytes from a donor can be fertilized and transferred to the carrier. Fresh embryos may be transferred when the cycles have been synchronized in the same way as that used for oocyte donation (see below) or the embryos may be cryopreserved for transfer into the carrier during a later cycle. Screening and testing for infectious agents from all gamete providers reduces the risk to the gestational carrier. Finally, psychological and legal counsel for all parties should be obtained before proceeding with a gestational carrier pregnancy.

## ART in cancer survivors

### Testing ovarian function

Ovarian reserve, a measure of the quantity of oocytes remaining in the ovary is difficult to assess accurately. It can be useful to test ovarian reserve prior to initiating reproductive treatment in cancer survivors since gonadotoxic treatments oftentimes reduce the primordial follicle pool and effectively "age" the ovary. Diminished ovarian reserve, even in young women, may already have been present prior to gonadotoxic treatments [11] and it will almost certainly be exacerbated after therapy. Early follicular phase follicle stimulating hormone (FSH) and estradiol (E2), inhibin B, antral follicle count, anti-Müllerian hormone (AMH) and response to ovarian stimulation (in addition to other measures) have all been utilized to estimate ovarian reserve and indirectly assess the chance for pregnancy.

However, results of ovarian reserve tests in women who have previously undergone gonadotoxic treatment or are undergoing treatment during the testing period must be interpreted with caution. Patients who have received chemotherapy in the past have been shown to have diminished ovarian reserve with abnormal elevations in FSH as well as lower AMH, inhibin B and antral follicle count [12, 13].

None of the current tests, either alone or in combination, can predict with certainty whether a pregnancy will be achieved. Therefore, it is not justified to withhold treatment from a properly informed patient. A cancer survivor who maintains some follicles and ovarian function should be given a chance to attempt conception without utilizing her cryopreserved

tissue – as long as she is advised of the presumed prognosis in doing so. By contrast, a patient who exhibits extremely high FSH levels, very low inhibin or AMH levels or has a negligible antral follicle count, may wish to discuss oocyte donation or other reproductive alternatives. If she has previously cryopreserved eggs or embryos, she should be encouraged to use the cryopreserved material.

## Ovarian stimulation

The physician treating a cancer survivor who wishes to have children will want to keep one central principle in mind: if the patient was treated with gonadotoxic agents or pelvic RT, no matter what her present age or ovarian function, she is likely to have reduced ovarian function earlier than her age would suggest. Therefore, as soon as she is ready, she should be encouraged to attempt conception. If she is not successful within 6–12 months, a thorough evaluation and intervention should be undertaken promptly. Finally, strategies to achieve pregnancy in the shortest time frame possible are reasonable, if not imperative, in this population.

For these reasons, we should recommend IVF for the infertile cancer survivor who fails to conceive a pregnancy after a limited trial of ovulation induction and intrauterine insemination (IUI). In vitro fertilization (IVF) offers the greatest chance for pregnancy in the shortest time interval. This is additionally important in women with estrogen sensitive tumors in order to minimize their exposure to multiple courses of ovarian stimulation.

For women who have stored gametes or embryos prior to gonadotoxic treatments, the following caveats are equally important: when ovarian function still exists and they are prepared to become pregnant, they should be encouraged to conceive either on their own or with ART. It should be emphasized that having frozen oocytes and embryos does not guarantee pregnancy success; rather they should be considered as the last option should the woman become sterile. One should not allow women who can still conceive to delay childbearing because of a false "sense of security" that their future reproductive potential is guaranteed by cryopreservation of either eggs or embryos. And, in general, and especially if the patient is considering having more than one child, consideration should be given to maintaining the cryopreserved eggs or embryos in reserve. If the patient has reasonable ovarian function and a normal fertility evaluation, she should attempt to conceive a pregnancy first in a natural cycle with timed intercourse. If she is unsuccessful after 3–6 months or if there is tubal occlusion or male factor, ART would be the best option. She gives herself the best chance of fulfilling her reproductive desires by retaining her frozen gametes or embryos until either her last attempt at pregnancy or her ovaries fail.

Patients with a history of cancer (not known to be hormonally sensitive) may be stimulated with gonadotropins in the same way as patients who are not cancer survivors. The main difference will be a generally lower response to gonadotropin stimulation – and an accompanying lower implantation and pregnancy rate – as compared to either age-matched controls with infertility or cancer patients prior to chemotherapy [14, 15].

Patients who received prior chemotherapy for a variety of cancer types have significantly lower AMH levels and fewer oocytes retrieved as compared to age-matched patients undergoing fertility preservation prior to chemotherapy. Additionally, the implantation rate for patients who had undergone prior chemotherapy was only 7.9%, significantly lower than expected for the infertile population in the same age group [16]. In a separate study, when a group of patients who had received local treatments for their cancer was compared to a group who had received systemic therapies, response to stimulation was better in patients who had received local treatment only. Additionally, the study suggested that the pregnancy rate in patients who had received systemic chemotherapy was lower than that of those who had received only local treatments [14].

Finally, these results suggest that a liberal embryo transfer policy should be applied. Patients should be counseled that their chances for pregnancy as well as the rate of multiple gestation, even with an increased number of embryos transferred, will be lower as compared to other infertile patients of the same age. Documentation of this counseling should be placed in the patient's record and the reason for exceeding the clinic's transfer guidelines for patients in that age group should be documented as well.

As in any other IVF situation, additional embryos can be cryopreserved. There is no reason to believe that cryopreserved good-quality embryos will not have reasonable survival rates; however, there is no specific data regarding outcomes with cryopreserved embryos in cancer survivors.

## Ovarian stimulation for hormonally sensitive cancers

Standard ovarian stimulation for ART results in high levels of circulating estrogen – potentially 10 times the peak levels of spontaneous cycles. Breast or endometrial cancer survivors and their doctors are appropriately concerned about the impact of these high estradiol levels on the risk of recurrence. Because of this theoretical risk, stimulation regimens that utilize estrogen-receptor modulators such as tamoxifen or agents which suppress estradiol synthesis (aromatase inhibitors) have been explored and may be preferable to conventional stimulation protocols.

Several alternative methods of ovarian stimulation have been described. These use estrogen receptor modulators or aromatase inhibitors, either alone or in conjunction with gonadotropins, in an effort to stimulate multifollicular development while reducing or avoiding high serum estradiol levels. Most protocols have been developed and studied in the context of stimulation of newly diagnosed breast cancer patients for fertility preservation. However, the protocols may have application for cancer survivors as well.

In a randomized controlled trial, our group compared tamoxifen, tamoxifen in combination with FSH or letrozole in combination with FSH, to stimulate follicle development (Figure 26.2). The highest number of total and mature oocytes was obtained in the letrozole/FSH group (11 ± 1.2 and 8.5 ± 1.6, respectively) compared to either the tamoxifen/FSH group (6.9 ± 1.1 and 5.1 ± 1.1 respectively) or the tamoxifen-alone group (1.7 ± 0.3 and 1.5 ± 0.3, respectively). Despite high peak estrogen levels in the tamoxifen, tamoxifen/FSH and letrozole/FSH groups (419 ± 39 pg/ml, 1182 ± 271 pg/ml, 405 ± 45 pg/ml, respectively), cancer recurrence rates were not different between groups after a mean follow-up of 554 ± 31 days [17]. The timing of final oocyte maturation with human chorionic gonadotropin (hCG) in tamoxifen or letrozole stimulation cycles is similar to protocols using clomiphene citrate: oocyte maturity is typically reached when the lead follicles are approximately 20 mm [18]. Additionally, when patients with breast cancer underwent ovarian stimulation for fertility preservation using letrozole and gonadotropins at our institution, there was no difference in disease-free survival compared to patients who elected to proceed directly to adjuvant treatments without ovarian stimulation [15]. Randomized studies with modifications

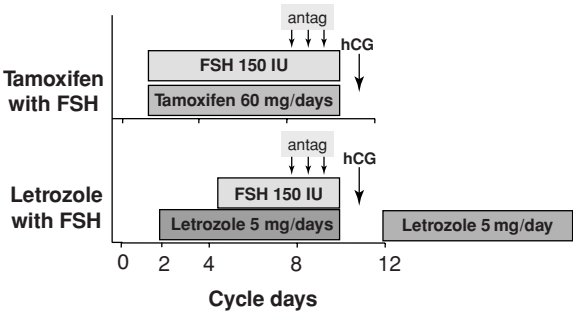


**Figure 26.2** Protocols for ovarian stimulation of the breast cancer survivor. antag, antagonist; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin.

to the above protocols are currently underway at our institution and others in an effort to further improve ovarian response and confirm safety. At this time, we can reassure patients with a history of estrogen-sensitive tumors that ovarian stimulation, using protocols that modulate the estrogen receptor or the ovarian hormonal response, do not appear to increase short-term recurrence. Further studies need to be performed to confirm and extend these findings.

Patients who are concerned about ovarian stimulation, either with or without modification, may express interest in natural cycle IVF. The success rates for these cycles are relatively low. Patients should be counseled about the possibility of cycle cancellation due to premature ovulation, failure to retrieve an oocyte or lack of an embryo to transfer. Additionally, the reduced viability of oocytes in patients who have undergone prior chemotherapy renders natural cycle IVF in this population questionable.

Another option, which has demonstrated some success in polycystic ovary (PCO) -like patients, is retrieval of immature oocytes after hCG with or without a truncated course of gonadotropin stimulation [19]. This field is reviewed in detail in Chapter 36. The significantly reduced developmental potential of embryos derived from in vitro matured oocytes (manifested in lower implantation rates and higher miscarriage rates), the need to transfer excess embryos in an attempt to maintain acceptable pregnancy rates and the requirement for prolonged hormone replacement in these women limits the present applicability of this approach for the cancer survivor [20].

As treatments for cancer improve and survival extends, more women of reproductive age who have been exposed to potentially gonadotoxic therapy will present to their physicians desiring pregnancy. Many

of these women will achieve a pregnancy spontaneously. However, for others, fertility potential will be reduced. In general, women who have been treated with gonadotoxic therapy will experience diminished ovarian reserve and menopause at earlier ages than their peers.

Women with ovarian function, but diminished ovarian reserve, can probably safely undergo ART and can be reassured that, so far, recurrence rates are no different when compared to women who elected not to attempt pregnancy. Stimulation with standard protocols for IVF or novel protocols in patients with hormonally sensitive tumors can be undertaken. However, the success rates in cancer survivors who received systemic chemotherapy or pelvic RT are not equivalent to their age-matched peers without a history of cancer treatment. Despite advances, some will not achieve genetic pregnancies. Some will choose to pursue adoption or child free living. Others may choose to pursue pregnancy through oocyte donation.

## Oocyte donation in cancer survivors

Egg donation is not suitable for everyone, and for some it will be morally, religiously or emotionally out of the question. However, for those for whom DE seems a reasonable, less stressful alternative, for those for whom methods of preserving genetic reproduction are not feasible or carry risks to survival and for those who carry a cancer predisposition gene that they prefer not to transmit, DE has much to offer. It has been with us for a quarter of a century and has a track record of success [21–23]. In the USA, for example, in 2006, 16 976 donor oocyte cycles were performed and 5393 or 32% of recipients achieved a delivered baby after a single fresh donor oocyte transfer [24]. The success rates are much higher – approaching 100% – if recipients undergo multiple attempts [25].

Depending on the cancer, its stage and grade and, for some cancers, the presence or absence of estrogen-receptor positive cells, there may be cancer survival risks associated with ART for fertility preservation. These hypothetical risks may be either as a result of the fertility treatments themselves or as a result of delay in cancer treatment. In most cases, the actual (evidence-based) risk to survival is unknown, further complicating informed consent and decision making for the young cancer patient.

In addition, some young women recently diagnosed with cancer will be carriers of cancer predisposition genes. Hereditary breast cancer syndromes (BrCa 1 and 2), familial adenomatous polyposis, multiple endocrine neoplasia syndromes, retinoblastoma and hereditary non-polyposis colon cancer syndrome are only a few examples [26, 27]. Without doubt, as we begin to understand the biology of cancer, there will be more in the future. We have already seen that some of these young people will wish to have children who are not carriers [28–31]. Technology – PGD with transfer of unaffected embryos only, or prenatal diagnosis with termination of affected fetuses – offers one set of solutions. However, these are added interventions into what is already very high tech, high stress reproduction. Both procedures carry risks and, when the gene is dominant, half of the embryos or fetuses so conceived will be carriers. Thus, for example, everything else being equal, a young woman, recently diagnosed with cancer, carrying a BrCa gene *and* wishing to have a non-affected child will find that the success rate for reproductive preventive care will be half that of a recently diagnosed cancer patient who is not a carrier. Some recently diagnosed cancer patients who carry predisposition genes will see DE as a simpler solution: a proven, highly successful way to build a family and, at the same time, eliminate a deleterious gene.

A young woman who has been diagnosed with cancer, along with other losses, may have lost confidence in the normal functioning of her body. A pregnancy – genetic or donor egg – offers her one of life's most normal – and yet most miraculous – bodily experiences. The chance to feel her body swell and fill is a life-affirming occurrence. Likewise, for the adoptive mother who delights in her baby's first smile. We do not mean here to minimize the cultural and emotional meaning of genetic reproduction. We simply mean to emphasize that, for some people, DE – or adoption or child-free living – is the best possible solution to a difficult situation.

All of this means that, even with a good prognosis for fertility preservation, some cancer patients may choose to demur. The young cancer patient faces physical and emotional ordeals, as well as a daunting array of difficult choices to make in an impossibly short time. Sometimes a plan for oocyte donation (or adoption or child-free living) is a better option than attempted fertility preservation. It is our hope that this chapter will help the advisors – oncologists, reproductive endocrinologists, psychologists and others – to support not only the patient who chooses to pursue

**Table 26.1** Mechanics of oocyte donation

| Donor | Recipient |
|---|---|
| *Recruitment* and screening | *Screening* |
| • Medical/infectious disease | • Medical |
| • Psychological | • Psychological/psycho-education |
| • Genetic | • Genetic screening of partner |
| • Informed consent | • Informed consent |
| *Matching* | *Matching* |
| • Ovarian stimulation | • Preparation/synchronization of endometrium |
| • Retrieval of oocytes | • Fertilization |
| | • Transfer |

fertility preservation but also the one who chooses to decline it.

To this end, we believe that it is important that we discuss oocyte donation right from the beginning, and sometimes even in some detail, along with other options for fertility preservation. Some patients may want to hear a lot about it, others may not. But, it is all part of an ultimately hopeful message that, while life may be different, it will go on.

## Clinical practice of oocyte donation

In the future, the majority of oocyte donation cycles may be done using cryopreserved and stored oocytes, similar to what is done in present sperm banks [32]. At the time of this writing, though, almost all oocyte donation is done using fresh oocytes, with the cycles of donors and recipients synchronized. The mechanics of oocyte donation thus involve a number of steps: the recruitment of suitable donors, informed consent of donors and recipients, matching of donors to recipients, ovarian stimulation of the donor and the retrieval of her oocytes. At the same time, we prepare the recipient's endometrium hormonally for transfer and synchronize it to the donor's stimulation cycle. Finally, we fertilize the retrieved oocytes with the appropriate sperm and transfer the resulting embryos into the recipient's prepared uterus (Table 26.1).

### Recruitment of donors

Third-party reproduction is one of the most ethically complex aspects of reproductive health care. Even societies with generally similar values, legislate donor recruitment very differently [33]. Thus, some countries have mandated anonymity (Spain, France, Belgium, Denmark) while others have mandated the *opposite* – that donors be identifiable to their genetic offspring (UK, Sweden, Austria, Switzerland, the Netherlands, Canada, New Zealand and the Australian state of Victoria) [34].

Some countries do not allow monetary compensation (UK) or strictly regulate it (in France donors can be compensated for documented expenses) while others regulate more loosely (Spain allows a small monetary compensation and expenses) [34]. In others, compensation is unregulated (USA, India), though it may be loosely limited by professional guidelines [35].

Some countries (Italy, Germany) do not allow oocyte donation at all. In others, oocyte donation is legal, but so strictly regulated as to make it impractical (China's "double blind" regulations, for example, have essentially eliminated oocyte donation in that country).

In general, these national regulations meet the needs of their respective societies. However, differences in policy have fostered trans-border reproductive care [36, 37]. Not only do recipients travel for oocyte donation services, but, also, centers actively recruit donors across national borders [38, 39]. Not surprisingly, generally, more prosperous countries recruit from less prosperous countries. For some donors, the small stipend, the travel, the meals and the hotel stay are enticing indeed, if not undue inducement.

In 2005, European IVF centers performed 11 475 oocyte donation cycles, or 3% of the 418 111 IVF cycles done in Europe that year [40]. By contrast, in the same year, US IVF centers performed 134 260 IVF cycles of which 16 161 or 12% were oocyte donation cycles [41]. This difference is striking. The explanations are

various, but include cultural differences, the lack of universal insurance coverage for infertility in the USA, the significant compensation paid to US donors, the predominance of anonymity in US gamete donation and, most significantly, much less US government regulation.

Specifically, US law allows both anonymous and known donation and the American Society for Reproductive Medicine (ASRM) guidelines suggest that both are acceptable [35]. Compensation is not legislated. A 2007 ASRM Ethics Committee Report set the following guidelines for compensation:

> Sums of $5000 or more require justification and sums above $10 000 are not appropriate …
>
> To avoid putting a price on human gametes or selectively valuing particular human traits, compensation should not vary according to the planned use of the oocytes (e.g. research or clinical care), the number or quality of oocytes retrieved, the outcome of prior donation cycles or the donor's ethnic or other personal characteristics [35].

Medical professionals screen and care for American oocyte donors. Many IVF programs recruit donors themselves. However, commercial, for-profit agencies (run mostly by business people, although sometimes by lawyers and, occasionally, by medical professionals) recruit a large, but difficult to quantify, proportion of American donors.

There are about 150 independent egg donor agencies in the USA. Only about a third have signed agreements to abide by ASRM guidelines regarding donor compensation. Furthermore, a recent review of 53 websites of the *assenting* agencies found that, despite their signed agreements, 24.5% advertise compensations that do not adhere to ASRM guidelines [43].

Thus, for many reasons, including both the relatively recent cultural pressure for delay of childbearing and the comparative ease of availability of egg donors, oocyte donation is a large and growing part of American fertility treatments. There are concerns about these developments on several fronts. Nonetheless, the availability and relative social acceptance of DE services is agreeable news for American cancer survivors.

### Anonymous versus known donation

At the present time, most oocyte donation worldwide is anonymous. However, family and known donation is the best choice for some recipients. This may be especially true for some cancer survivors. A sister, cousin or friend may have good reason to decide to donate to a survivor and may derive great satisfaction from the action. For the recipient, the opportunity to have a child who is genetically related to her family (a sister or cousin donation) or the kindness of the gift can also make it a good choice.

By contrast, family and known donation also carries higher risk of coercion and higher risks of complicated family dynamics. This may be particularly true when the recipient is a cancer survivor. For these reasons, we recommend psychological screening of all involved parties – prospective donor, recipient and their respective partners – before proceeding with known donation [35, 44].

### Infectious disease screening of donors

By law, donors in the USA must be screened for risk factors for infectious diseases as well as tested for exposure to specific infectious diseases according to detailed Food and Drug Administration (FDA) guidelines [35, 45]. While the risk of infectious disease transmission is remote, FDA regulations have provided a standard of care for this aspect of the screening of US gamete donors.

### Genetic screening

By contrast, genetic testing of US donors is not legally mandated. The appropriate professional society sets the standard of care: ASRM recommends that all donors be tested for cystic fibrosis (CF) mutations and that additional testing be based on risk factors identified by history. According to ASRM guidelines, donors and their first-degree relatives should be free of mendelian disorders, major malformations, significant familial diseases with a known genetic component and mental retardation of undocumented etiology [35].

Genetics is progressing rapidly and recommended screening tests change very quickly. The American College of Medical Genetics has published excellent guidelines regarding ethnicity and population-based genetic screening (http://www.acmg.net). Of course, the absence of legal mandates does not decrease US physicians' responsibility to act in the best interests of the donor, the recipient parents and the potential child. Of note is that screening gamete donors is not the same as preconceptual testing in women attempting pregnancy. The testing of young, often unmarried oocyte donors and the dissemination of information so acquired carry different ethical, medical and

psychological implications than those same tests in prospective parents. If at all possible, a genetic counselor should counsel every prospective oocyte donor before testing. Ideally, it should also be a genetic counselor who conveys the test results and their often complex implications [46].

In our practice, a genetic counselor meets with the prospective donor at the time of her initial visit. The counselor takes a detailed family health history, counsels the prospective donor about the implications of testing and then advises about screening tests and any increased risk detected. Over more than a decade, we have seen that a battery of tests does not substitute for a professional genetic counselor. A good genetic counselor will identify issues that are detectable only through careful, directed history or, in some cases, observation of physical characteristics [46–48]. Genetic health issues may be mild in the donor herself or her family but may be of variable penetrance and may have serious consequences for potential offspring.

At the time of the writing of this book, our practice is to screen all donors for CF, fragile X, spinal muscular atrophy (SMA), and a chromosome analysis. We do other testing based on the donor's ethnic background. Examples would be thalassemia testing for donors with Mediterranean background or a panel of 15 tests for donors with Ashkenazi Jewish background. Although ASRM guidelines suggest that recipients who have been appropriately consented can accept a donor who is a carrier for a recessive disease [35], the idea is controversial. It is our practice not to accept such young women as donors, even if the male partner of the prospective recipient is negative for the identified disease. We, and others, are concerned about the difficulty of informed consent in these often complex situations where as-yet unidentified mutations (or *de novo* mutations) could result in potentially severe disease despite negative male partner screening [47].

In addition to screening the donor, we routinely test the male partner of the recipient for CF, SMA and all diseases indicated by his ethnic background. If the male partner is found to be a carrier, additional testing of the donor may be indicated and the recipient family should be counseled about implications of the findings.

Finally, we need to advise recipients not only of the genetic evaluation findings, but also of their limits. Only a relatively small number of genetic diseases are amenable to detection through either history or testing. Birth defects are common in the population at large – 3–4% of all births – and most of these defects are not screenable [46].

We see meticulous genetic screening and counseling as part of our ethical responsibility to the families who come to us, to the donors and to the donor-conceived persons who may result from our care.

### Psychological screening

Psychological evaluation of the donor and the recipient and, when applicable, their families can be helpful to all involved. Third-party reproduction is a complex action with potential long-term repercussions. Participants have both the right and the responsibility to explore the consequences and emotions that accompany these decisions. We can listen, explore feelings, support and educate donors and recipients in a way that helps all parties to make choices that are right for them.

#### Donor

The donor should be free of significant psychiatric pathology. The psychologist should assess current life stressors, traumatic past events (including any unresolved history of abuse or neglect) and coping skills. Psychosocial history should include such items as family history, interpersonal relationships, educational background, employment history, sexual history and any history of past or present substance abuse. Current and past prescribed psychoactive medication use should also be assessed.

Informed consent will probably be formally obtained at another point in the evaluation. However, the psychological interview provides a valuable moment to ascertain that the prospective donor is aware of the procedures involved and of their potential risks.

The donor should be participating without coercion. If her donation is anonymous, we can give her information about program precautions that are taken to protect her anonymity. In addition, we want to apprise her of the limits of anonymity in a complex technological society and in a world in which values and laws change rapidly [35].

When the donor is known, it is, perhaps, even more important that situations of coercion be identified and that the prospective known donor be supported by the medical team if she is uncomfortable with egg donation. In family situations, in particular, and even more so when the prospective recipient is a cancer survivor,

coercion can have many faces. It can be intergenerational, it can threaten family membership and it can stir up long dormant feelings of anger and resentment [44].

Even when the prospective donor is happy that she can be of help, there are details to be discussed. Both families should have discussed and come to agreement about disclosure (and timing of disclosure) to the donor-conceived person(s) and to other family members, including genetic half siblings. The cancer prognosis should be openly and truthfully discussed among family members. In cases where the prognosis is poor, painful as it is to do, a discussion should be held about how relationships might change were the recipient to die.

#### Recipient

Psychological consultation can be very helpful for all recipients of donated oocytes and is specifically recommended for recipients considering known donation [44, 49]. These sessions are, at least partly, "psychoeducation."

The psychologist and recipient family can discuss feelings about pregnancy, positive and negative aspects of disclosure and the family can be provided with a bibliography of children's and adult books related to ovum donation. Hopefully, any differences between the partners in attitudes towards disclosure can be resolved well in advance of the birth of a child.

Additionally, the session offers the recipient and her partner the opportunity to discuss their feelings about the loss of the hypothetical genetic child that they might have made together. Couples often carry many fears and fantasies about gamete donation, including concerns that the donor will try to reclaim the offspring or that the child will wish to seek out his/her "real" mother. Most recipient mothers wonder how they will feel carrying another women's genetic child and whether they will bond to that child in utero, after birth and throughout his or her lifetime. In situations where either blame or guilt has colored the process, these feelings, too, can be discussed. To the extent that resentment of the process might later surface as resentment towards the child, these conversations are helpful steps towards building a healthy family.

In the case of recipients who are cancer survivors, other issues and feelings may be layered onto the decision for DE. Anger and resentment about the cancer and its treatment, seemingly resolved, may resurface. And, while a pregnancy may restore confidence in the normal function of the body, a DE failure may renew feelings of physical inadequacy.

In conclusion, we do psychological screening of donors and recipients in order to address a number of different issues and to protect all parties involved. Our goal is to help the recipient and the donor navigate a complicated psychological terrain. By doing so, we hope that we will build stronger families and fulfill some of our responsibilities as advocates for the donor-conceived person.

### Matching of donors and recipients

Although there has been little scientific inquiry into the criteria that make for success in families created with donated gametes, current practice has widely included phenotypic matching. Thus, most programs worldwide include an attempt to match donors and recipients for criteria such as coloration, height and ethnic background. And, in fact, most recipients (and donors) express interest in some degree of phenotypic matching.

However, in general, European donor oocyte programs are less concerned about phenotypic matching than American ones. In addition, several societal trends suggest that phenotypic matching may assume a lesser role in the future. First, adoption practices have increasingly relinquished proscriptions against interfaith and interracial adoption [50, 51]. Some of these historical proscriptions have come to seem old-fashioned. Secondly, there is an increased tendency for, and increased professional advice in favor of, disclosure of means of origin to individuals conceived through gamete donation [35]. Thirdly, there is a growing interest (some of it legally mandated) towards identity-release donation. These trends decrease the need for secrecy that may have been a part of phenotypic matching. Finally, families have become more diverse and the concept of a family has become more diverse. All of these tendencies together may lessen the importance of phenotypic matching in the future.

Nonetheless, at the present time, most recipients (and many donors) express interest in at least some degree of phenotypic matching.

### Care of the oocyte donor

The oocyte donor is a young healthy person who takes on risks and receives no direct benefits from them. From an ethical point of view, she is more like a research subject than a patient [52, 53]. It is the guiding principle of this section to emphasize that the aim of

the physician caring for the oocyte donor should be to reduce the rate of complications to as near zero as possible, while still maintaining reasonable success rates for the recipients.

Ultimately, if we commodify our donors, if we do not treat them with the care and respect to which they are entitled, we will lose the confidence of the public, of the recipients and of the donors themselves. Caring well for donors is both a public trust and a professional duty.

## Ovarian stimulation of the oocyte donor

Increased success in oocyte donation, as in IVF generally, is linked to an increased number of healthy mature oocytes that can be retrieved and fertilized. To achieve this goal, physicians override two basic physiological controls. First, in the early follicular phase of the natural cycle, negative feedback of estrogen and peptides on the pituitary results in a steadily decreasing secretion of FSH. It is the exposure to this decreasing concentration of FSH which allows a single dominant follicle to emerge from the cohort of recruited follicles. By contrast, in stimulated cycles, exogenous gonadotropins override the negative feedback of rising estrogen and allow multiple follicles in the recruited cohort to mature.

Secondly, the rising levels of estradiol in a natural cycle will trigger a positive feedback response at the pituitary resulting in a surge of luteinizing hormone (LH), which will trigger ovulation. If not controlled, the rising levels of estradiol in the stimulated cycle may trigger the LH surge prematurely, resulting in immature oocytes. Thus, stimulation cycles for IVF include either a gonadotropin-releasing hormone (GnRH) agonist or antagonist to prevent premature LH surges. Because the endogenous trigger for ovulation has been suppressed, an exogenous trigger must be given at the appropriate time. Usually, this exogenous trigger is hCG. However, for reasons that will be discussed, we may find advantages for the donor in the use of a GnRH agonist to trigger an endogenous pituitary LH surge.

Stimulation of donors is, in most ways, similar to the stimulation of infertile IVF patients. However, the stimulation of donors differs in two important ways. First, by definition, donors are young, healthy and presumably fertile. Thus, they generally respond briskly, have a higher risk of early onset ovarian hyperstimulation syndrome (OHSS) and need to be stimulated more gently than most IVF patients. Secondly, and more importantly, donors experience risk but achieve no benefit from their stimulation cycles. The donor's risks include OHSS, ovarian torsion, bleeding, infection and anesthesia complications, amongst others. When all care is taken, the risks are low – reported as $<1\%$ – but may be serious [54–57]. In worst-case scenarios, a complication may affect the ongoing well-being and fertility of a healthy young woman.

### Dual suppression

In order to minimize OHSS, stimulation protocols should utilize relatively low doses of gonadotropins and should include appropriate "step down" regimens once the cohort of follicles has been recruited. One such protocol, commonly used in patients at high risk for OHSS, and highly appropriate for donors, is a dual suppression with oral contraceptive pills (OCPs) and GnRH agonist overlap [58]. Because the OCP cycle length can be varied, this protocol in donors has the additional advantage of simplifying synchronization with the recipient cycle (Figure 26.3 [59]).

### GnRH agonist trigger for ovulation

Over the last few years, increasing evidence suggests that the incidence of OHSS can be significantly decreased by the use of antagonist suppression along with a GnRH agonist trigger without compromising oocyte or embryo quality [60]. Human chorionic gonadotropin has a dramatically longer half-life than LH (2.3 days versus 21 min) [61, 62], and therefore generates a prolonged luteotrophic effect. This is beneficial in the IVF patient (it is the corpus luteum that prepares the endometrium and sustains the early pregnancy), but results in risk without benefit in the oocyte donor. Specifically, early GnRH agonist trigger studies in IVF patients seemed to result in lower pregnancy rates, which, in retrospect, were almost certainly related to early luteolysis and lack of support of the endometrium [63]. By contrast, when the early pregnancy was appropriately supported with estrogen and progesterone, comparable pregnancy rates were achieved with GnRH trigger as compared to hCG [64].

Because the donor herself has no early pregnancy to support, she may be the ideal candidate for agonist trigger protocols. Not surprisingly, two recent large prospective studies of these protocols in donors have shown significant decreases in OHSS without any compromise in recipient pregnancy rates [65, 66]. As physicians gain more experience with agonist trigger

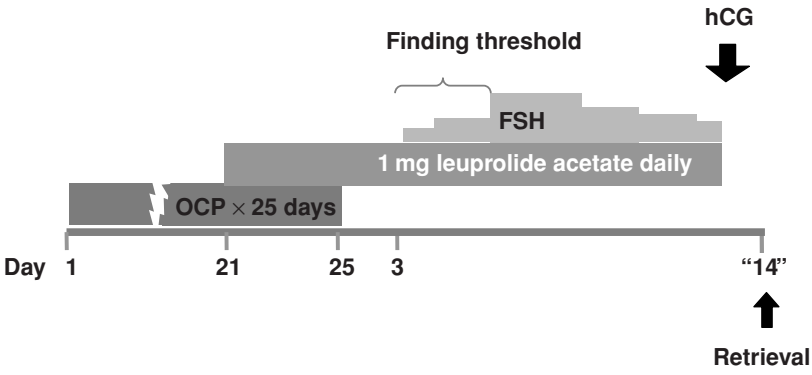


**Figure 26.3** A dual suppression protocol used by us in 1986, still valid today. FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; OCP, oral contraceptive pill. Reprinted with permission from Rosenwacks [59], courtesy of Elsevier, ©1987 American Society for Reproductive Medicine.

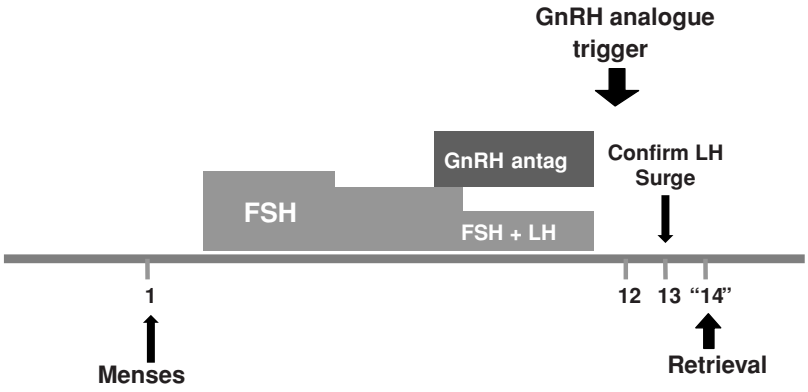


**Figure 26.4** A GnRH agonist trigger protocol from our program today. antag, antagonist; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

protocols, these protocols may become standard of care for oocyte donor stimulation (Figure 26.4).

### Oocyte retrieval

Donor oocyte retrieval does not differ from oocyte retrieval for IVF. Under conscious sedation, and using a transvaginal ultrasound-guided needle, the follicles are aspirated and the oocytes identified in the follicular fluid. Complications of the procedure include infection and intraperitoneal bleeding, as well as anesthetic complications. All complications are rare and yet potentially serious.

The incidence of pelvic infection following transvaginal ultrasound-guided oocyte retrieval ranges from 0 to 1.3% [67, 68]. In a review of 2670 oocyte retrieval procedures, Bennett *et al*. reported 18 cases resulting in infection (0.6%), 9 of which were severe with pelvic abscess formation (0.3%) [56]. The severity of infection can range from a minor infection with pyrexia, leukocytosis and abdominal pain, to a major medical event such as pelvic abscess formation or sepsis. Signs and symptoms can appear within hours to days after the retrieval in an acute infection, while pelvic abscess may not present clinically for several weeks. Pelvic infection is a particularly concerning complication for a young egg donor because even a relatively minor event, especially if not treated promptly, may result in infertility later in life.

Minimizing infectious complication involves careful initial screening and testing for any history of pelvic inflammatory disease or sexually transmitted disease, meticulous preparation of the vagina with povidine-iodine, attention to making the fewest vaginal punctures possible, and, possibly, prophylactic antibiotics at the time of retrieval.

Intraperitoneal bleeding is another rare but potentially serious complication. Bleeding can arise from inadvertent puncture of an arterial vessel. More commonly, it is probably secondary to generalized oozing from the highly vascularized ovaries, similar, but

in exaggerated form, to the intraperitoneal bleeding that can occur with natural ovulation. Clinical observations suggest that intraperitoneal bleeding, like OHSS, occurs more commonly when more oocytes are retrieved. This is physiologically plausible: the larger, more highly vascularized ovaries and the increased number of follicular punctures would likely predispose to bleeding.

### Screening of the recipient

A cancer specific evaluation, as outlined in the first part of this chapter, should be done before any other screening of the recipient. Only with this cancer-specific evaluation complete, do we move on to routine preconception screening, including infectious disease screening, blood type, thyroid stimulating hormone (TSH), rubella and varicella titers. In addition, we screen the male partner for infectious disease and ask him to provide a sample for semen analysis. He should be counseled and screened for recessive genetic disease. As discussed previously, genetic evaluation of the male partner has benefits even when the donor has been screened.

Although some controversy exists about the importance of a mock or preparatory cycle for most recipients [69], recipients who have been previously treated with pelvic irradiation, as discussed in the first section of this chapter, should have particular attention paid to the function of the uterus and should have a preparatory cycle done. In these cases, a mock replacement cycle with evaluation of the endometrial response to hormonal treatment by ultrasound measurement of endometrial thickness and endometrial biopsy can be very helpful. It will alert us to situations that require individualized estradiol dosing to prepare the endometrium and will highlight cases that should be considered for single embryo transfer. In some cases, uterine evaluation will show that oocyte donation is too risky to be entertained. The patient will need to choose between a gestational carrier for the donated oocytes and other alternative ways to form a family.

### Preparation and synchronization of the recipient's endometrium

In the natural cycle, events in the endometrium are physiologically synchronized to events in the ovary. The steadily increasing serum levels of estradiol – produced by the ovary as the oocyte matures – result in proliferation of endometrial glands and stroma and the development of endometrial LH receptors. Following ovulation, granulosa cells in the dominant follicle, now the corpus luteum, continue to produce estrogen, but now also produce increasing amounts of progesterone. The endometrium responds with tightening and coiling of glands and blood vessels and with the accumulation and secretion of glycogen into the endometrial cavity. This is, not coincidentally, the ideal environment for the support of the early embryo. The embryo first migrates freely in the cavity, making use of the rich glycogen secretions, and then begins implantation into the endometrial wall, seeking and finding vascular support from the abundant, coiled vasculature.

Many cancer survivors seeking DE treatment have little or no ovarian function. For those with ovarian failure we can proceed directly with estrogen and progesterone, while for those patients who exhibit ovarian function, we use GnRH analogues to shut down pituitary stimulation of the ovary before beginning steroid replacement. In either case, there will be no follicles to prepare the endometrium and no corpus luteum to support the early pregnancy. Instead, we must supply estrogen and progesterone exogenously. In doing so, we seek to mimic both the preparation of the endometrium and its synchrony with the developing embryo.

In general, preparation of the donor oocyte recipient's endometrium has remained unchanged since the inception of egg donation. Figure 26.5 shows a replacement protocol used by the author in 1986 [59].

At the time, three ongoing pregnancies from IVF oocyte donation had been reported in the USA and only a few others worldwide. Amazingly, in a field in which so much has changed so quickly, this protocol from 25 years ago could be used, essentially unchanged, to teach a patient or train a new nurse today.

Basically, estradiol, delivered either as oral micronized estradiol or through a transdermal patch, is used to initiate proliferation of the glandular and stromal cells of the endometrium. Our current practice is to use slowly increasing doses of estrogen delivered via transdermal estrogen patches. The patches have the advantage of being relatively physiological; estradiol is delivered slowly and continuously and the hepatic "first pass" is avoided [70]. We usually start with a dose of 0.1 mg/day, increase this to

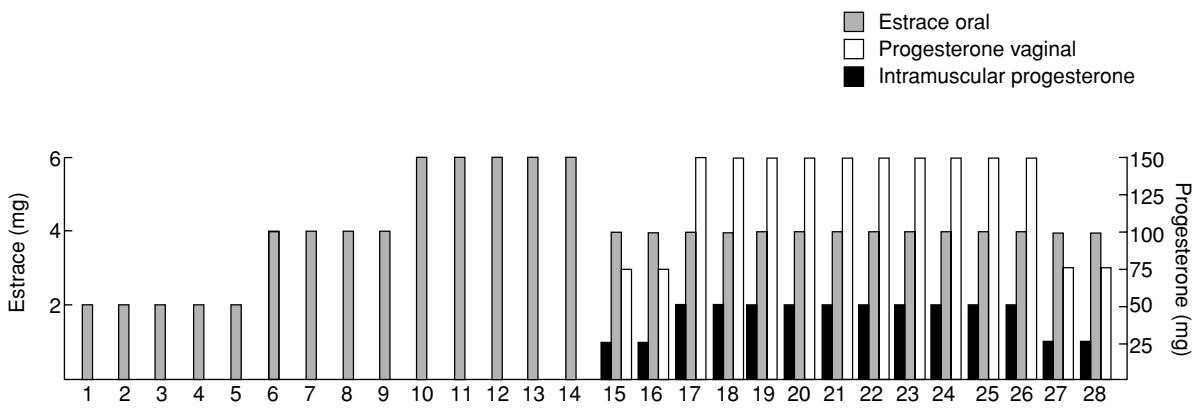


**Figure 26.5** A replacement protocol used by us in 1986, still valid today. Reproduced with permission from the American Society for Reproductive Medicine.

0.2 mg/day as the donor begins her gonadotropin stimulation, and increase to 0.4 mg/day for approximately 4 days prior to the donor's ovulation. During the luteal phase, patches are decreased back to 0.2 mg/day [71). Most patients tolerate the patches well – although redness or rash at the patch site may be seen in up to 1% of patients. As in the natural cycle (where follicular phase length may vary considerably without affecting fertility), there is considerable latitude in the length of the estrogen-only phase of the replacement cycle [72, 73]. This is fortunate; without this flexibility, synchronization would be difficult.

Progesterone replacement, however, is a different matter. Its administration must be synchronized closely to the donor's cycle. The presence of adequate endometrial levels of progesterone at the correct time in the cycle is critical both for the preparation of the endometrium for implantation and for the support of the early embryo [73].

Progesterone can be administered either by the vaginal route or as intramuscular injections of progesterone in oil [74]. Intramuscular progesterone has the disadvantage of being irritating to muscle; patients often experience pain at the injection site and occasionally develop fever and leukocytosis. On the other hand, serum levels can be reliably used to monitor and titrate the dose, which is not true of the better-tolerated vaginal suppositories. (Oral micronized progesterone is variably absorbed and is rarely used for recipient replacement cycles.)

In our practice, we begin progesterone on the day after the donor receives her ovulatory trigger at a dose of 2 mg/day (im) or, more rarely, 300 mg/day (suppository). On the following day, we double the dose and continue it either until the pregnancy test is negative or, if positive, until the placenta takes over steroidogenesis, usually by 10 or 11 weeks of pregnancy [75]. If intramuscular progesterone is used, we monitor serum levels and adjust the dose to keep the serum concentration above 20 ng/ml. The estradiol dose is titrated to yield a serum concentration between 100 and 200 pg/ml (Figure 26.6).

## Conclusion

A young cancer patient may find significant psychological benefit in preserving oocytes or embryos prior to embarking on a grueling course of chemotherapy. This is true even if she ultimately does not need to use the cryopreserved tissue. Many times, especially if she is young, ovarian function will return following treatment. However, even those survivors with regular menses, may find that ovarian reserve and egg quality have been compromised. The cancer survivor with regular menses needs to understand this and, after a safe interval, be encouraged to attempt conception as soon as she is ready. Physicians should discuss the advantages of an abbreviated evaluation for a couple unable to conceive within a few months. Often, effective intervention will shorten the time to pregnancy. Sometimes, ART is the best initial approach.

When conception does not occur and when prevention has not been done, or has not been successful, a cancer survivor may consider pregnancy through

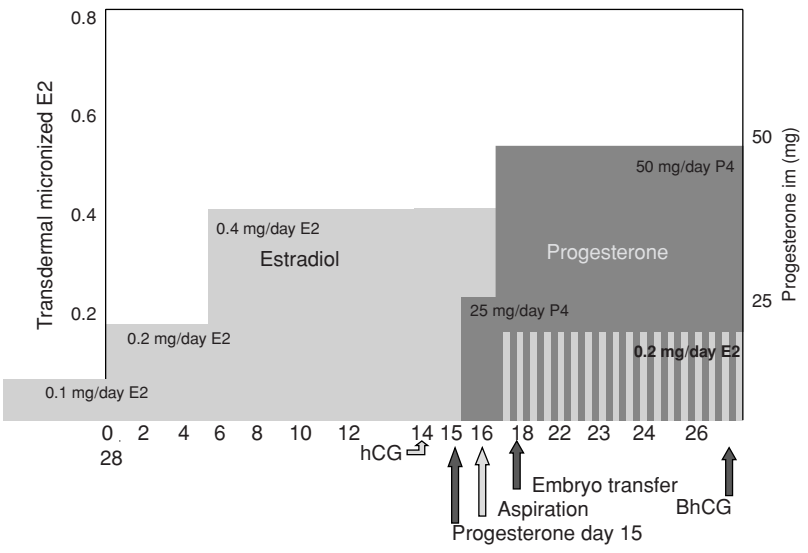


**Figure 26.6** A similar replacement protocol from our program today. BhCG, beta human chorionic gonadotropin; E2, estradiol; hCG, human chorionic gonadotropin; im, intramuscular; P4, progesterone.

oocyte donation. As long as physicians screen both donors and recipients meticulously, use techniques to minimize risk to donors and are careful about informed consent to both parties, pregnancy through oocyte donation can be a positive experience for all involved.

Finally, we have an understandable desire to be optimistic and encouraging to our young cancer patients. However, these patients also need support as they adjust to the very real losses that accompany a cancer diagnosis. The losses range from a loss of innocence and invulnerability, to the physical losses that accompany surgery, chemotherapy and radiation, to changes in life expectations, including potential loss of reproductive options, and, finally, to the potential loss of life itself. A focus on technological solutions alone for quality of life issues may deny the newly diagnosed cancer patient the opportunity to reconsider values and to put life events in perspective.

We know that genetic reproduction is important to living things, humans included, but we also know that the genetic imprint that an individual makes is erased in a few generations. This chapter addresses, among other things, issues that occur when preservation of fertility has not been possible or has not been the best choice for a given individual. We can help our patients by expanding their options and choices, but we can help them also by affirming that there are many paths to a fulfilled and satisfying life.

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Chapter 27

# General overview of ovarian cryobanking

S. Samuel Kim

## Introduction

Fertility preservation is now recognized as the most essential quality of life issue in young cancer survivors. Although several strategies to preserve fertility in women have been developed, most of them are still experimental. Ovarian cryobanking, either freezing ovarian tissue or the whole ovary, is currently perceived as a promising technology for fertility preservation which draws enormous attention not only from scientific communities but also from the general public.

Although still in its developmental stage, ovarian tissue cryopreservation followed by transplantation has proven to be successful in many animals. Furthermore, we have been witnessing the successful restoration of fertility after ovarian transplantation in humans since 2004. To date, 14 healthy babies have been born worldwide after transplantation of frozen–thawed ovarian tissue [1–7].

It is exhilarating to see the steady progress and increasing enthusiasm for clinical applications of this technology. However, ovarian cryopreservation and transplantation should remain experimental until the efficacy of this technology is proven. Indeed, there are numerous technical and ethical issues that should be resolved with this technology. In this chapter, three urgent and critical problems involved with ovarian tissue cryopreservation and transplantation (cryoinjury, ischemic tissue damage, cancer cell transmission) are discussed. In addition, the current status of human ovarian tissue transplantation and whole ovary transplantation by vascular anastomosis are briefly addressed.

## Synoptic history of ovarian transplantation

The history of ovarian transplantation dates to the eighteenth century. Although many animal experiments were performed in nineteenth-century Europe, the first human ovarian tissue transplantation was reported by Robert Morris in New York in 1895 [8]. By 1901, Morris had performed 12 ovarian transplantations (autograft as well as allograft). In 1906, he claimed a live birth after autografting ovarian tissue to the broad ligament of a 33-year-old woman with polycystic ovary syndrome.

The discovery of cryoprotectants (CPAs) in London in 1948 was a scientific breakthrough which made it possible to cryopreserve living cells and tissue. Just after discovery of CPAs, there was a flurry of experiments on freezing gonadal tissue followed by transplantation. In 1960, restoration of fertility was reported after orthotopic isografting of frozen–thawed ovarian tissue in oophorectomized mice [9]. Over the next 30 years, however, there was no further progress in this field. In 1994, Gosden *et al*. succeeded in restoring fertility in sheep after autotransplantaion of frozen–thawed ovarian tissue, which rekindled the interest in this technology with new perspectives, especially as a potential strategy to preserve fertility in cancer patients [10]. Ten years later, in 2004, the first baby was born after orthotopic autotransplantation of cryopreserved human ovarian tissue in a woman with Hodgkin's lymphoma [1].

Transplantation of the whole ovary with vascular anastomosis is not a new procedure either. In 1906, Alexis Carrel in New York, who later won a Nobel

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Prize, reported the first ovarian transplantation by vascular anastomosis in cats. Since then, successful transplantation of the whole ovary with microanastomosis of vascular pedicles has been reported in many animals including dogs, cats, rodents, rabbits, sheep and primates [11–13]. In 1987, Michel Leporrier in France reported the successful heterotopic transplantation of the whole ovary with vascular anastomosis before pelvic irradiation to treat Hodgkin's disease [14], the first successful whole ovary transplantation (heterotopic) in humans. In 2009, the first baby was born as a result of orthotopic transplantation of the intact ovary by vascular anastomosis between monozygotic twins [15]. Indeed, the surgical complexity of vascular anastomosis is no longer a barrier of human ovary transplantation. The real challenge of vascular transplantation of the whole ovary is perfecting cryotechnology for organ cryopreservation. The first success in restoring fertility after vascular transplantation of the cryopreserved whole ovary was achieved in 2002 in rats [16].

## Clinical guidelines for ovarian cryobanking

It is important to provide full counseling before any procedures to protect patients and to prevent misuse of technology. As ovarian tissue banking is not yet an established technology, the current status and experimental nature of the technology should be fully and accurately explained. At the same time, some details of ovarian tissue banking should be discussed, including the surgical procedure and its risks, efficacy of freezing and storage, and options of future use of cryopreserved tissues. In addition, it is imperative to communicate with the patient's oncologist before and after the procedure.

The physical and psychological conditions of the patient should be evaluated and considered before the procedure. The age of the patient is another crucial factor to consider as the chance of restoration of ovarian function and fertility is closely correlated to the number of follicles in the ovarian graft. Current experiences with human ovarian transplantation suggest that women over 40 years of age may not be good candidates for ovarian tissue banking as the chance of fertility restoration after transplantation is extremely low [17].

Nevertheless, advanced reproductive age cannot be an absolute indicator for low ovarian reserve in view of individual variations. It is therefore recommended that ovarian reserve is assessed with endocrine tests as well as pelvic ultrasound (antral follicle count) to guide clinical decision making. The serum follicle stimulating hormone (FSH) level has been used widely to assess ovarian reserve, but its accuracy to predict ovarian reserve is limited. In my opinion, the single best test to assess the ovarian reserve before ovarian tissue banking (especially in cancer patients) is the serum anti-Müllerian hormone (AMH) level as it is a direct assay for ovarian reserve (since it is produced from granulosa cells of the ovarian follicles) and can be tested any time of the menstrual cycle (unlike FSH).

The safety of transplanting stored ovarian tissue is crucial as the risk of re-introduction of cancer cells exists in certain cancers. At present, the type of malignancy, the type of treatment and the prognosis after treatment should all be considered to determine if the candidate is suitable for ovarian cryobanking. To date, autotransplantation of ovarian tissue in Hodgkin's lymphoma patients appears to be safe [17]. While patients with Hodgkin's lymphoma are indeed good candidates for ovarian banking, the types and doses of chemotherapeutic regimen should be considered before offering ovarian cryobanking. The chance of losing fertility with an ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine) regimen in young patients with Hodgkin's lymphoma is $<$15%, which cannot justify routine use of ovarian tissue banking in this population. Furthermore, ovarian tissue banking should be discouraged in patients with systemic or disseminated malignancies.

Ovarian tissue banking will be most useful for patients who need to undergo hematopoietic cell transplantation, since the risk of premature ovarian failure is extremely high due to highly gonadotoxic preparatory regimens. The use of hematopoietic cell transplantation is no longer limited to leukemia and lymphoma but has been extended to solid malignant tumors, such as breast cancer, and non-malignant conditions, such as lupus, rheumatoid arthritis, aplastic anemia and sickle cell disease.

As the efficacy and safety of ovarian tissue banking has not been established and there is no consensus for indications for ovarian tissue cryobanking, for the time being it is prudent to offer this technology selectively to women who are at a high risk of losing their fertility and with a good long-term prognosis.

**Table 27.1** Challenging issues with human ovarian tissue cryopreservation and transplantation

| |
|---|
| • Patient selection criteria |
| • Cryoinjury/optimization of freezing technique |
| • Safety issues/prevention of cancer cell re-introduction |
| • Ischemic-reperfusion injury |
| • Effective graft sites |
| • Effective in-vitro follicle culture technique |
| • Quality of oocytes matured in a graft |
| • Efficacy for restoration of fertility |
| • Ethical issues, especially in children |

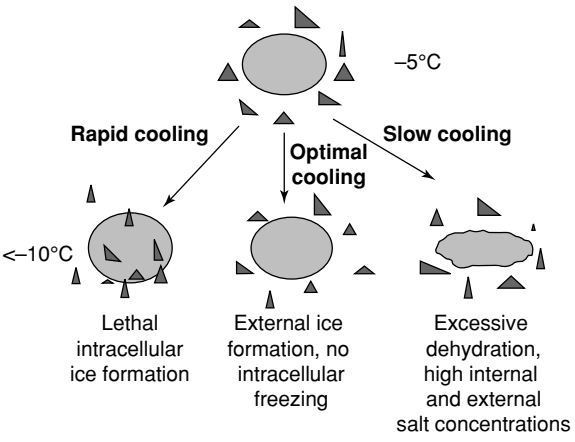



**Figure 27.1** Effects of cooling rates during cryopreservation of living cells.

## Challenging issues with ovarian tissue cryopreservation and transplantation

The landscape of ovarian tissue transplantation has been changed since 1994. It is no longer staying in the research arena but entering into the clinical realm. We have solid evidence that the strategy of ovarian tissue cryopreservation followed by autotransplantation works to restore fertility in cancer patients. Nevertheless, this strategy contains numerous technical and scientific problems as well as ethical issues (Table 27.1). Of these, three challenging issues (cryoinjury, ischemic tissue damage and cancer cell transmission) are discussed in this chapter.

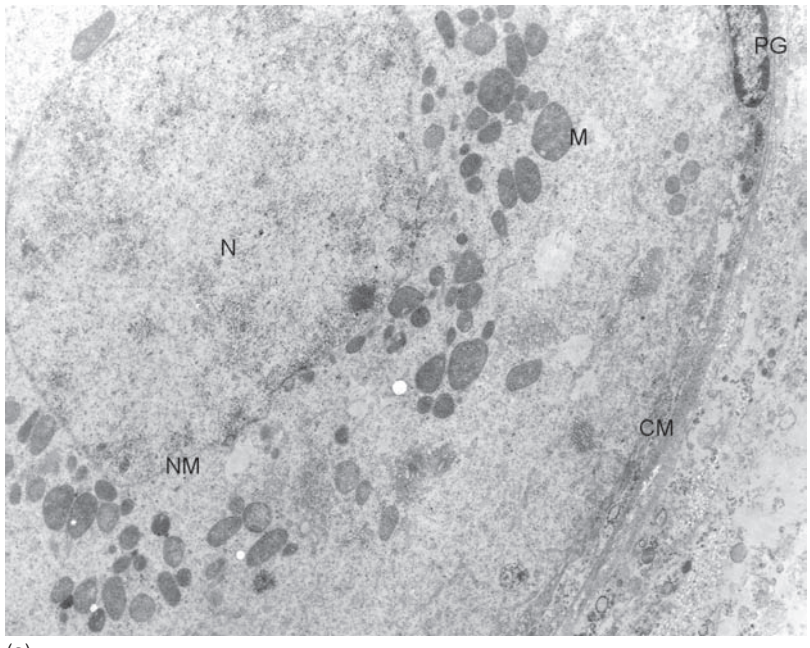
### Cryoinjury

Cryopreservation of ovarian tissue by slow freezing and rapid thawing is successful (50–80% follicle survival rates), but current methods are still not perfect and require further optimization to minimize the loss of follicles and ovarian function. Two main mechanisms of cryoinjury are intracellular ice formation and salt deposits. The most damaging phase (increased ice formation and growth) of slow freezing is during cooling between –10 and –40°C, especially when the liquid phase is supercooled. However, significant cryoinjury can occur during the thawing (re-expansion) phase because of changes in the composition of the surrounding milieu, possibly mediated by temporary leakage of the plasma membrane [18]. Indeed, the thawing rate is important in maintaining cell viability. Newton and Illingworth noticed the higher follicle survival and in-vitro maturation rates when samples were thawed at 27°C rather than at 37°C in a mouse model [19].

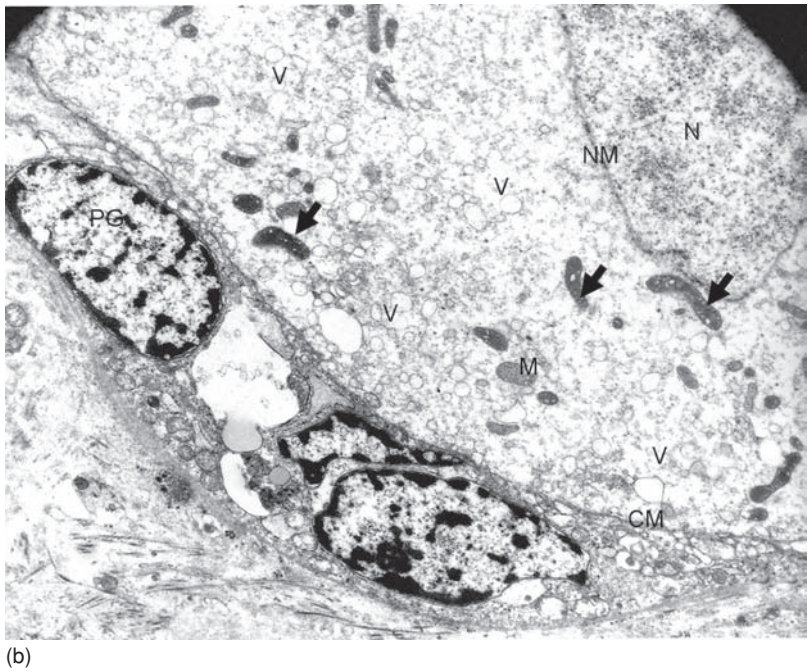
To minimize cryoinjury, cooling rates need to be fast enough to reduce the exposure of cells to high intracellular concentrations of electrolytes, but they should be slow enough to dehydrate cells and avoid intracellular ice formation (Figure 27.1). The thawing rates should be fast enough to prevent formation and growth of ice crystals. Cryopreservation of living cells requires CPAs, which can be cytotoxic. The toxicity of CPAs depends on the inherent characteristics of the chemical itself, duration of exposure and temperature.

It is much more difficult to optimize freezing and thawing conditions for tissue compared with those for isolated cells. Tissue is composed of various cell types with different physical parameters that influence cryostability during cooling and CPA penetration. Naturally, longer exposure time to CPAs increases the toxicity. Furthermore, extracellular ice formation is as detrimental as intracellular ice in multicellular systems. Nevertheless, almost two-thirds of immature follicles survive in human ovarian tissue after slow freezing and rapid thawing [20, 21]. The majority of these follicles are morphologically normal by light microscopy, but distinctive ultrastructural changes can be detected in frozen–thawed tissue by electron microscopy (e.g. mitochondrial and membrane damage, vacuoles in the cytoplasm) (Figure 27.2a,b).

Vitrification with a high concentration of CPAs and ultra-rapid cooling, in which the aqueous phase turns directly into a solid amorphous phase, can be advantageous for tissue freezing as it can eliminate both intra- and extra-ice formation, which directly affects the




(a)




(b)

**Figure 27.2** Ultrastructural changes in human primordial follicles before (a) and after (b) cryopreservation of ovarian tissue (slow freezing) detected by transmission electron microscopy (TEM). (a) A primordial follicle from fresh ovarian tissue showing intact nuclear and cell membranes. Normal-shaped mitochondria are clustered around the nucleus. (b) A primordial follicle from frozen–thawed ovarian tissue showing extensive vacuolation (V) throughout the cytoplasm. Nuclear and cell membranes are still intact, but mitochondrial damage is evidenced by dilated cristae in the mitochondria (arrows). CM, cell membrane (oolemma); M, mitochondria; N, nucleus; NM, nuclear membrane; PG, pre-granulosa cell; V, vacuoles.

survival of cells in ovarian tissue. However, there are limitations to tissue cryopreservation by vitrification. A key limiting factor of vitrification is the toxic effects of CPAs (chemical and osmotic). The high concentrations of cryoprotectant required for vitrification necessitate a short equilibration time to minimize the toxicity. Unlike individual cells, tissue requires a longer exposure to high concentration of CPAs to reach optimal CPA penetration. This is a dilemma for vitrification of ovarian tissue. The antifreeze proteins

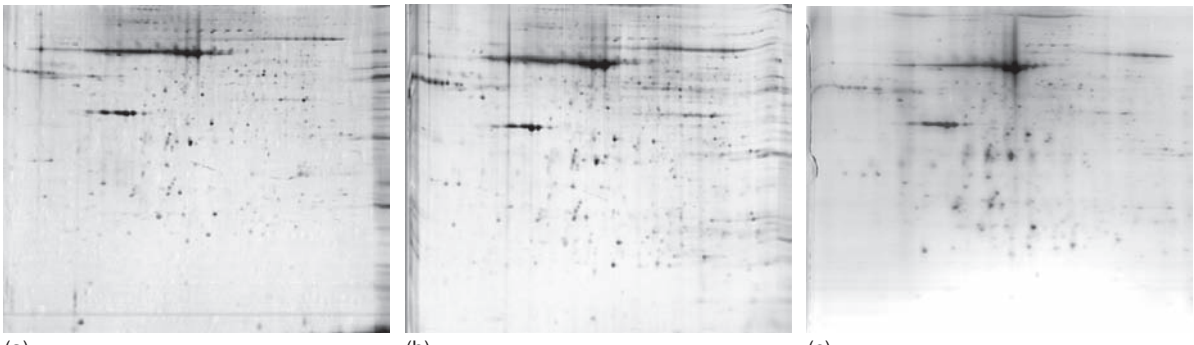



**Figure 27.3** Protein expression patterns assessed by two-dimensional gel electrophoresis in: (a) fresh ovarian tissue; (b) slow frozen–rapidly thawed ovarian tissue; (c) vitrified–warmed ovarian tissue.

(non-colligative CPA) that inhibit ice-nucleating events may reduce the toxicity of CPAs by allowing lower concentrations of CPAs to be used for vitrification. The second factor that can compromise tissue survival is re-crystallization at warming (devitrification). Complete elimination of devitrification is not easy unless very high concentrations of CPAs are used. The probability of devitrification can be significantly reduced by the warming rate equal to those imposed during the cooling.

To date, there is no standard vitrification protocol and it is rather confusing to see the many variations, which include types and concentrations of CPAs, durations and steps of equilibration, methods of cooling (straws, grids, aluminum foils, Cryovials, solid surface vitrification, direct plunging), warming temperatures and solutions. Detailed discussions are in Chapter 28. Overall, we see more favorable results with the use of both permeating and non-permeating CPAs for vitrification (e.g. 20–40% of dimethyl sulfoxide and ethylene glycol as permeating CPAs and sucrose or trehalose as non-permeating CPAs), a two-step or multi-step equilibration, direct contact to liquid nitrogen, high warming temperature (37–40°C) and serial dilution in solutions with sucrose. Our study showed that vitrification of bovine ovarian tissue after equilibrating in 5.5 M ethylene glycol for 20 min at room temperature was as effective as slow freezing [22]. Currently, we have adopted a two-step equilibration method using 20% dimethyl sulfoxide and 20% ethylene glycol as colligative cryoprotective agents for vitrification of ovarian tissue.

Although we have accumulated some knowledge of cryoinjury after freezing and thawing, cryotechnology for ovarian tissue cannot be perfected without further basic research on cryobiology, especially at the molecular level. As a part of studies investigating molecular and biochemical changes with cryopreservation, we have analyzed the protein expression in ovarian tissue between the fresh, slow cryopreserved and vitrified group before and after transplantation using two-dimensional gel and mass spectrometry technologies (Figure 27.3). By comparing the protein spots with significant intensity differences between samples, we could identify the proteins of significance including RAB4B, actin, Chain A and B (lectin), serpinb 1a protein, 33 laminin receptor homolog and glutathione-S-transferase. Of note, these proteins are related to tissue survival and metabolism [23].

## Ischemic injury

It has been proven that autotransplantation of frozen–thawed human ovarian tissue can restore endocrine function as well as fertility. However, the follicular loss in the grafted ovarian tissue is unacceptably high, and it is mainly caused by tissue hypoxia after grafting while waiting for angiogenesis. In rodents, ovarian tissue slices become revascularized within 2–3 days after grafting [24]. If the ischemic period is longer than 24 h, irreversible hypoxic tissue damage is unavoidable in the ovarian graft [25]. Primordial follicles are more resistant to ischemia than growing follicle or cortical stromal cells. Nevertheless, most primordial follicles die of ischemia rather than of cryoinjury, and only between 5 and 50% of follicles survive after grafting [26, 27]. The future of ovarian tissue transplantation depends on the development of new strategies to

facilitate angiogenesis or to protect the graft from ischemia (especially within 24 h after transplantation). Indeed, many researchers have begun to investigate different strategies to minimize ischemic injury in the ovarian graft, such as applying antioxidants and angiogenic factors [23].

Nugent *et al.* demonstrated that antioxidant treatment using vitamin E improved the survival of follicles (45% in the control group versus 72% in the treated group) 7 days after transplantation [28]. In addition, the vitamin E supplemented group showed a significant reduction in lipid peroxidation in ovarian grafts on day 3 after grafting. The results of this study indicate that antioxidants can reduce damage from lipid peroxidation during ischemia in ovarian grafts.

Our study also demonstrated that ascorbic acid, an antioxidant, can effectively protect bovine ovarian grafts from hypoxic damage [25]. In this study, we measured the rates of oxygen consumption and apoptosis as parameters of tissue damage after incubating ovarian tissue at 37°C for different time periods (1, 3, 24 and 48 h of ischemia) with or without ascorbic acid. The significant tissue damage was evidenced by the decrease in the oxygen consumption rate and the increase in apoptosis after 24 h of ischemia, and antioxidant treatment significantly reduced apoptosis in ovarian cortical stroma.

Ovarian tissue is endowed with abundant genes for angiogenic factors including vascular endothelial growth factors (VEGF), transforming growth factors (TGF), fibroblast growth factors (FGF), and angiopoietins. Expression of these genes is stimulated by hypoxia through hypoxia inducible factors (HIF) that regulate transcription of key angiogenic growth factors. There are many factors that can stimulate angiogenesis. One of the factors that can be clinically useful is gonadotropin, as gonadotropins stimulate angiogenesis by upregulating the angiogenic growth factors including VEGF and angiopoietin.

Imthurn *et al.* showed that exogenous gonadotropin could increase the number of developing follicles by facilitating angiogensis, but the magnitude of the effect was influenced by the timing of the gonadotropin administration relative to the time of grafting [29]. They found that gonadotropin injection started at or after surgery was not effective. To maximize the number of follicles after grafting, gonadotropin stimulation should be started 2 days before surgery and continued 2 days after transplantation.

To investigate the effect of exogenous administration of gonadotropin on angiogeneisis after ovarian grafting, we evaluated gene expression of angiogenic growth factors including HIF-1, VEGF, TGFβ and angiopoietin-1, 2 and 3 in rat ovarian grafts 2, 7 and 30 days after transplantation. The mRNA expression of HIF-1 and angiopoietin-1 were low on day 2 but increased on days 7 and 30. Angiopoietin-2 and 3, VEGF and TGFβ were increased from day 2 and peaked on day 30 after transplantation. When we compared the VEGF expression and apoptosis in the ovarian graft between the gonadotropin-treated and control groups, we did not find any added benefits of exogenous gonadotropin for angiogensis or follicular development in ovariectomized animals [30]. Nevertheless, a significant increase in the VEGF188 isoform in the gonadotropin-treated group suggested the positive role of gonadotropin in the early stage of angiogensis in ovarian grafts. Of note, the mRNA expression of VEGF188 increased on day 2 and returned to normal on days 7 and 30. On the other hand, VEGF164 and VEGF120 showed no changes on days 2 and 7 but increased on day 30.

The optimal graft sites should be further determined to minimize ischemic damage and to improve follicular survival after avascular ovarian grafting. It is reasonable to expect better graft survival when ovarian cortical tissue is grafted to vascular sites, such as muscle tissue or kidney capsule, rather than subcutaneous tissue. Furthermore, the importance of vascular smooth muscle cells and pericytes in sustaining vascular and tissue integrity after transplantation has been demonstrated [31]. Perhaps, the secure strategy to prevent ischemic damage is using whole ovary transplantation with vascular anastomosis. However, cryopreservation of the whole human ovary along with its vascular pedicles is a huge technical challenge.

## Cancer cell transmission

The risk of cancer cell transmission is a serious safety issue related to ovarian autotransplantation in cancer patients. Shaw *et al.* reported that healthy AKR mice that received ovarian grafts from donor mice with lymphoma died of the same disease within 2–3 weeks after transplantation [32]. However, Kim *et al.* demonstrated the safety of transplanting human ovarian tissue from lymphoma patients using a xenotransplantation model; human ovarian tissue harvested from 18 lymphoma patients with high-grade disease was

xenografted to non-obese diabetes/severe combined immunodeficient (NOD/LtSz-SCID) mice [33]. None of the animals grafted with ovarian tissue from lymphoma patients developed disease, whereas all positive control animals that received lymph-node sections containing non-Hodgkin's lymphoma cells developed human B-cell lymphoma. To date, there is no sign of relapse in more than 10 women with Hodgkin's lymphoma who underwent autotransplantation of cryobanked ovarian tissue worldwide [17].

Ovarian metastasis is clinically rare in most cancers of young people, and its risk depends on the disease type, activity, stage and grade. The chance of ovarian metastasis of Wilms' tumor or Hodgkin's disease is negligible, whereas the risk of minimal residual disease (MDR) in ovarian tissue from leukemia patients is a real concern. Indeed, MDR in the ovarian tissue from a chronic myelogenous lymphoma (CML) patient has been detected by highly sensitive real-time polymerase chain reaction (RT-PCR) for BCR-ABL transcript [34]. It is thus imperative to screen ovarian tissue thoroughly for MDR before transplantation using sensitive markers to prevent re-introduction of cancer cells. Currently, the available methods to detect MDR include histology/cytology, immunohistochemistry, flow cytometry and PCR. Preoperative imaging can detect disease in the ovaries and prevent unnecessary surgery and storage. To date, there is no reported case of cancer recurrence due to autotransplantation of frozen–thawed ovarian tissue, which should not be interpreted as the proof of the safety. Indeed, it is too premature to assess the risks of cancer recurrence involved with this procedure.

## Current status of human ovarian tissue transplantation

There are three strategies, at least in theory, to mature follicles in frozen-stored ovarian tissue: autotransplantation; xenotransplantation; and in-vitro culture (Figure 27.4). Recently, significant progress has been made in immature follicle culture techniques. In particular, three-dimensional culture techniques and two or three-stage culture strategies are promising (see Chapter 32). There are, nevertheless, many variables and obstacles to overcome before perfecting these culture methods for clinical applications.

Although full development of human oocytes can be achieved after grafting ovarian tissue in the animal host (xenotransplantation), its clinical application is problematic because of safety and ethical issues. Grafting stored ovarian tissue back to the patient's own body (autotransplantation) therefore appears to be the most practical strategy in the clinical setting. In spite of skepticism, the first baby was born in 2004 after orthotopic autotransplantation of frozen–thawed ovarian tissue in a woman with Hodgkin's lymphoma. This is another milestone in the history of human ovarian transplantation, and it validates the clinical feasibility of ovarian transplantation for fertility preservation.

### Orthotopic autotransplantation

Although its efficacy should be further probed, restoration of fertility by orthotopic autotransplantation has been demonstrated in humans as well as in many animals. For orthotopic transplantation, ovarian tissue can be either transplanted onto the remaining ovary or into the peritoneal pocket of the fossa ovarica. It appears that grafting ovarian tissue in or onto the remaining ovary has advantages and more likely results in natural conception, unless the size of the ovary is too small as a result of atrophy [35]. In the <5 years since the first report of a live birth in 2004, the total number of babies born after orthotopic transplantation of frozen–thawed ovarian tissue has increased to 11 (including unpublished data). It is still too early to determine the efficacy of this technology, but the current data is encouraging: 6 live births out of 12 pregnancies following 30 transplantations and several ongoing pregnancies as of the end of 2008 [17]. Six pregnancies occurred spontaneously and five by in vitro fertilization (IVF) and embryo transfer. In the IVF group, 15 embryos (including 2 blastocysts) were transferred, which resulted in 6 pregnancies (2 miscarriages, 1 ectopic pregnancy and 3 live births). The details of orthotopic autotransplantation of ovarian tissue is discussed in Chapter 29.

### Heterotopic autotransplantation

Heterotopic autotransplantation is an attractive alternative to orthotopic autotransplantation as it can avoid invasive procedures and make the recovery of oocytes easy. In particular, it is a practical and cost-effective technology when repeated transplantation is required, because of the shortened life span of the ovarian grafts; or a hostile pelvic environment due to previous radiation; or severe pelvic adhesions precluding orthotopic transplantation. The duration of ovarian function

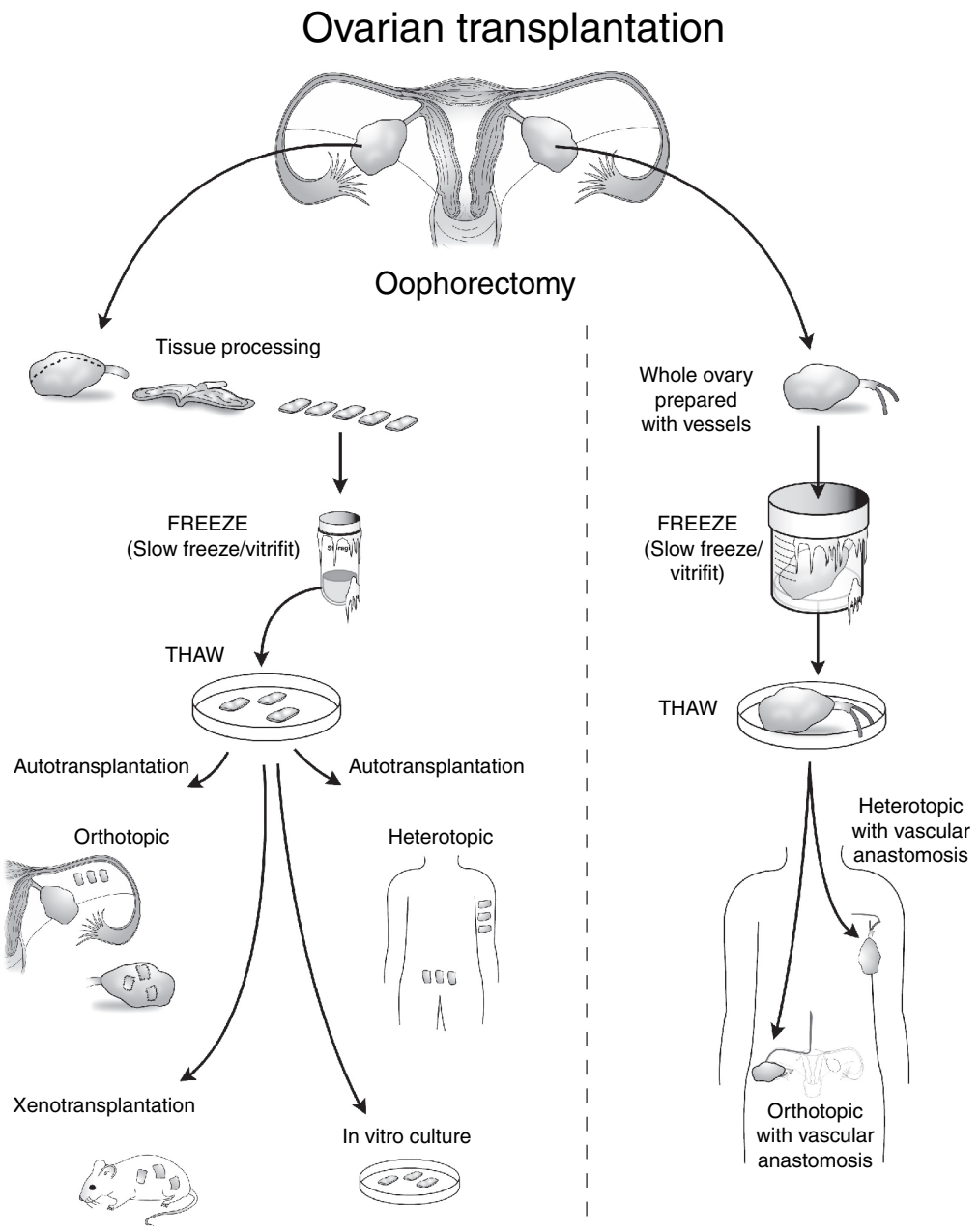



**Figure 27.4** Theoretical strategies for oocyte maturation in cryopreserved ovarian tissue and intact ovary. To date, the only strategy that produced live births is orthotopic autotransplantation of ovarian tissue. See plate section for color version.

after heterotopic transplantation of human ovarian tissue varies widely (between 3 and 60 months).

In theory, the optimal site should be rich in blood supply, convenient to implant, easy for oocyte recovery and mimic the surrounding environment of the ovary. Various heterotopic sites have been tested, which include subcutaneous tissue of the abdomen [36], forearm [37] or hip [23], rectus muscle [38], breast tissue [38], uterus [D. Nugent, unpublished data], as well as subperitoneal tissue beneath the abdominal fascia [39]. Nevertheless, the optimal site for heterotopic transplantation of human ovarian tissue is still elusive.

**Table 27.2** The advantages and disadvantages of heterotopic transplantation

| Advantages | Disadvantages |
|---|---|
| • Convenient for repeated multiple transplantations | • In vitro fertilization (IVF) procedure required |
| • Non-invasive procedure | • Efficacy not proven (no live birth yet) |
| • Easy access for oocyte retrieval without anesthesia | • Suboptimal (unknown) environmental effects on follicle growth and maturation |
| • Feasible for patients with severe pelvic adhesion | • Possible poor quality oocytes |

Albeit frozen–thawed ovarian tissue can be easily transplanted to the heterotopic site, obtaining healthy oocytes for IVF is a tremendous challenge. In fact, we noted compromised follicular growth and poor quality of oocytes retrieved from heterotopic ovarian grafts, which may be caused by the suboptimal environmental factors for follicle development such as temperature, local pressure, paracrine factors and blood supplies.

The re-establishment of endocrine function as well as oocyte retrieval after heterotopic transplantation of human ovarian tissue has been demonstrated by several investigators [37–41]. Nevertheless, no baby has been born after heterotopic transplantation. In 2004, Oktay *et al*. reported a case of embryo development from the oocytes retrieved from ovarian tissue implanted beneath the skin of the lower abdomen in a woman with breast cancer [40]. A total 20 oocytes were recovered (after 8 egg retrievals), but only 8 oocytes were suitable for IVF. Of these, only one fertilized normally and developed to a four-cell embryo, but no pregnancy ensued. In 2006, a Danish group reported a biochemical pregnancy after transferring a four-cell stage embryo which was generated by intracytoplasmic sperm injection (ICSI) following the retrieval of metaphase-II (MII) stage oocytes from ovarian tissue grafted to the heterotopic site [39]. In this case, frozen–thawed ovarian tissue was grafted to a midline subperitoneal pocket on the lower abdominal wall as a heterotopic site.

Over the past 8 years, Kim *et al*. transplanted frozen–thawed ovarian tissue heterotopically in cancer patients to assess the long-term ovarian function and restoration of fertility [41]. Four study patients (3 with cervical cancer, 1 with breast cancer), with an age range between 28 and 35 years, were identified and consented for heterotopic transplantation. All ovarian tissue had been cryopreserved using a slow-freezing method before cancer treatment. Heterotopic ovarian transplantation (to the space between rectus muscle and fascia in the abdomen) was performed between 2002 and 2005 (Figure 27.5). The re-establishment and maintenance of ovarian function was confirmed by serial blood test (FSH, luteinizing hormone [LH], estradiol, progesterone, testosterone) and ultrasound monitoring. Three patients were stimulated with gonadotropin followed by oocyte retrievals to investigate the restoration of fertility. The retrieved oocytes were matured in vitro and fertilized with partner's sperm.

The hormonal profile of all four patients was consistent with the postmenopausal level before transplantation. The return of ovarian function was evidenced by the elevation of serum estradiol levels and by the decrease of FSH levels below 20 mIU/ml between 12 and 20 weeks after transplantation in all 4 patients. However, restored ovarian function lasted only 3–5 months, and 3 patients (except 1 with relapsed disease) agreed to undergo a second transplantation. The return of ovarian function after the second transplantation was faster in all 3 patients (between 1–3 months) (Figure 27.6).

In contrast to first transplantation, we observed the establishment of long-term ovarian function (lasting for 9–60 months) after the second transplantation. All 3 patients maintained the FSH levels below 15 mIU/ml during this period. We were able to retrieve 7 oocytes (2 germinal vesicle [GV], 4 MI, 1 MII) from ovarian grafts in 2 patients between August 2003 and November 2006. Three of four MI oocytes were developed to full maturity in vitro. Four oocytes at the MII stage were fertilized and cultured in vitro for 2 or 3 days before cryopreservation. Currently, 4 embryos (at 6-cell, 3-cell, 2-cell and pronuclear [PN] stage) are stored in liquid nitrogen. Although these results are encouraging, it is difficult to predict whether it could be a clinically practicable technology since the

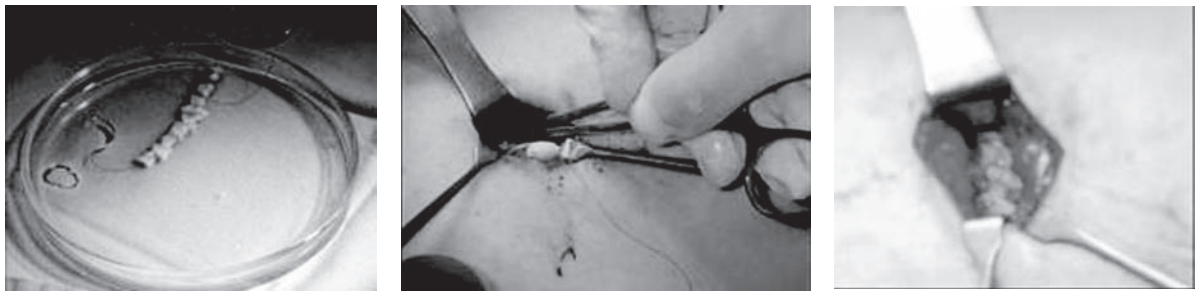


**Figure 27.5** The process of heterotopic transplantation of frozen–thawed human ovarian tissue to the space between the rectus muscle and the rectus sheath. Reproduced with permission from Elsevier. See plate section for color version.

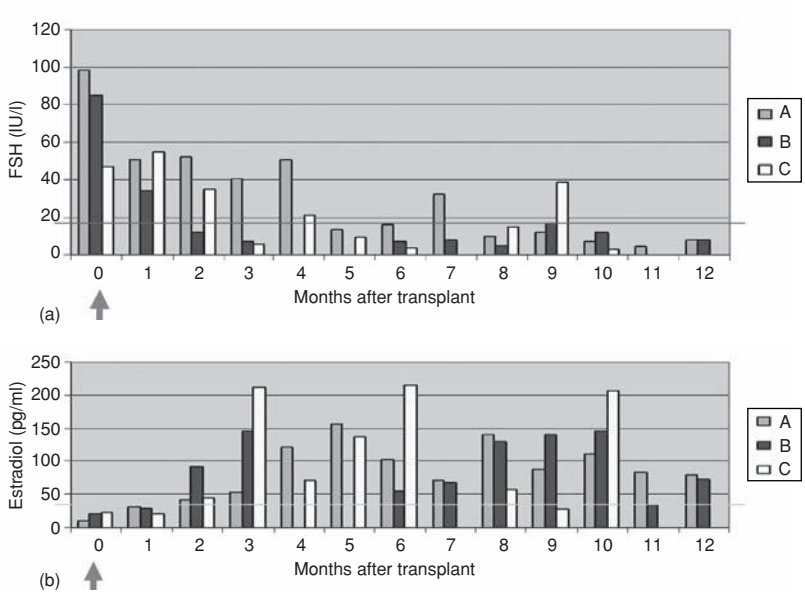



**Figure 27.6** Monthly follicle stimulating hormone (FSH) (a) and estradiol levels (b) after the second transplantation of ovarian tissue in three cancer patients (A–C). Estradiol production from ovarian grafts was noticed 2 months after transplantation, and serum FSH levels decreased below10 mIU/ml in 2 patients 3 months after transplantation. Reproduced with permission from Elsevier. See plate section for color version.

environment of heterotopic sites may not be as favorable for normal follicle development.

## Xenotransplantation

Numerous animal and human studies support the value of xenotransplantation of ovarian tissue as a strategy to preserve fertility and to conserve rare and endangered species. It has already been demonstrated that xenotranplantation of ovarian tissue from cat, wombat, sheep, African elephant, monkey and human to immunodeficient mice can support follicular development up to the antral stage [23]. Furthermore, generation of live young from xenografted rodent ovaries proved that it is a valid technology for animal conservation as well as fertility preservation [42].

Historically, xenotransplantation of human ovarian tissue was explored as a strategy to restore fertility in cancer patients with a high risk of cancer cell re-introduction with autotransplantation. Successful follicular development, ovulation and corpus luteum formation in the human ovarian tissue xenografted after cryopreservation have been demonstrated [43, 44]. Furthermore, Kim *et al.* succeeded in retrieving human oocytes that had developed in the frozen–thawed ovarian tissue xenografted to SCID mice [45]. All host animals were stimulated with gonadotropin from 20 weeks after xenografting. Grafts were recovered 36 h after human chorionic gonadotropin (hCG) administration. Fifty-seven grafts from 30 animals (total 60 grafts) were recovered, and 12 oocytes were collected from 26 antral follicles (size larger than

**Table 27.3** The use of xenotransplantation of human ovarian tissue for studies

| |
|---|
| • Angiogenesis |
| • Ischemic tissue damage |
| • Follicular biology and physiology |
| • Oocyte quality |
| • Steroidogenesis |
| • Minimal residual disease (MRD) of cancer |
| • Gonadotoxicity of various agents |

2 mm in diameter). After in vitro maturation for 36 h, only 2 oocytes were matured to the MII stage. Most oocytes retrieved from xenografts, however, showed aberrant microtubule organization and chromatin patterns.

This study indicated that immature oocytes in ovarian grafts can grow to maturity, but obtaining fully competent and healthy oocytes for fertilization can be problematic. We can speculate that the compromised follicular development and oocyte quality may be influenced by multiple factors such as cryoinjury, ischemic damage or in vitro maturation. However, most of all, the environment of the xenograft site, which is very different from that of the human pelvic cavity, may not provide optimal conditions for human follicular growth and maturation.

Even if we can overcome these technical difficulties, clinical application of xenotransplantation of human ovarian tissue will remain problematic until the safety and ethical issues are resolved. Nevertheless, xenotransplantation of ovarian tissue will continue to provide a useful experimental model to study follicular development, angiogenesis, ischemic tissue damage, MRD of cancer, gonadotoxicities of various agents and much more (Table 27.3).

## Whole ovary transplantation by vascular anastomosis

As an alternative to ovarian tissue transplantation, whole ovary transplantation has been explored. In theory, whole intact ovary transplantation with vascular anastomosis can restore the full function of the ovary. The main challenge of whole ovary transplantation for fertility preservation is the development of effective cryotechnology for the whole organ. The whole human ovary is vulnerable to cryoinjury, as it is bulky and composed of various cells and tissues. Unless we develop the optimal crytechnology for the organ, extensive cryoinjury to the ovary while freezing and thawing is unavoidable, and leads to the destruction of numerous cellular ultrastructures and, subsequently, their function. In particular, vascular injury with freezing and thawing is of great concern.

When freezing multicellular systems, extracellular ice can be as lethal as intracellular ice. In fact, there has been no documented success in freezing vital organs (such as livers and kidneys) because of the difficulty of preventing extracellular ice formation, particularly the formation of intravascular ice. Additional factors also make cryopreservation of the organ difficult, such as delivery of adequate concentrations of CPA evenly into all cells in a timely manner.

Nevertheless, it may be beneficial to transplant the whole ovary by vascular anastomosis as it should theoretically minimize ischemic damage. Many studies, however, showed that the follicular survival rate after whole ovary transplantation is no better than that of ovarian tissue transplantation. Courbiere *et al.* reported successful microsurgical transplantation of the fresh intact ovary, but follicular survival rate was only 6% [46]. This poor follicular survival is most likely due to prolonged warm ischemia time and significant thrombosis.

The results of transplantation of the frozen–thawed whole ovary are even more disappointing. Microvascular anastomosis of the whole cryopreserved ovary in sheep revealed large fibrotic areas with an absence of follicles (30–50%) in the transplanted ovary. As a consequence, the follicular survival rate was <8% [47]. This severe follicular loss may be caused not only by direct cryoinjury to the follicles due to suboptimal cryotechnology but also by ischemia induced by thromboembolism in the vascular system of the ovary after transplantation.

Viability data following whole bovine ovary cryopreservation have shown a significant detrimental effect of cryopreservation on the extent of arterial endothelial cell layer detachment and arterial smooth muscle damage [48]. In fact, Bedaiwy *et al.* demonstrated poor long-term vascular patency after autotransplantation of intact frozen–thawed bovine ovaries with microvascular anastomosis [49]. The anastomosed vessels were completely occluded in 8 of 11 cases, leading to immense follicular loss.

In summary, the main advantage of transplantation by vascular anastomosis is that it can restore blood supply immediately after transplantation. By minimizing ischemia time with whole ovary transplantation,

**Table 27.4** Advantages and disadvantages of whole ovary transplantation

| Advantages | Disadvantages |
|---|---|
| • Immediate blood supply to the graft | • Technical difficulties of organ cryopreservation |
| • Minimizing tissue ischemia | • Surgical complexity and longer surgical time |
| • Potential long-term ovarian function | • Ischemia-reperfusion injury |
| | • Thromboembolism |
| | • Increased morbidity(and mortality) |
| | • Potential risks of cancer recurrence (especially with minimal residual disease in ovarian medulla) |

greater longevity of ovarian function can be expected. On the contrary, many disadvantages of vascular transplantation of the frozen–thawed whole ovary are evident (Table 27.4). First, no reliable and successful cryopreservation for whole organ freezing is available, and it will take a while before perfecting this technology. Second, ischemia-reperfusion injury cannot be avoided even with vascular transplantation. Indeed, surgical time can be prolonged due to the technical complexities of vascular surgery, which can accelerate follicular loss through warm ischemia. Moreover, thromboembolism after vascular transplantation can cause extensive cell death and be life threatening under certain circumstances. Finally, whole ovary transplantation can increase cancer cell recurrence to a greater degree if the risk of residual ovarian medullary pathology is high, whereas it is unlikely with transplantation of small pieces of ovarian cortical sections.

## Conclusions

Ovarian tissue cryobanking has rapidly become a promising strategy for fertility preservation in cancer patients, and its use will extend beyond women with cancer. Although healthy babies have been born with orthotopic autotransplantation of frozen–thawed ovarian tissue worldwide, its clinical efficacy and practicability for fertility preservation is still unclear. There are many unresolved issues with ovarian tissue cryopreservation and transplantation, such as cryoinjury, ischemic injury, risks of cancer cell transmission and its efficacy. Heterotopic autotransplantation may be an alternative method to restore fertility with use of cyrobanked ovarian tissue. To date, there is no report of a live birth after heterotopic transplantation of ovarian tissue in humans, and its clinical practicability is questionable. However, it is too premature to abandon this strategy without further investigations, as heterotopic transplantation has several advantages over orthotopic transplantation.

Due to the limited life span of ovarian tissue transplants, many investigators have been exploring the feasibilities of whole ovary transplantation by vascular anastomosis. To date, no significant advantage of transplanting the whole intact ovary has been demonstrated, whereas the risks involved with the whole ovary transplantation by vascular anastomosis (especially after freezing and thawing) appear to be higher than expected. The safety and efficacy of these emerging technologies should be carefully examined and tested before full clinical application. Detailed discussions on ovarian cryobanking and transplantation are found in Chapters 28–31.

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Chapter 28

# Ovarian tissue cryopreservation

Debra A. Gook and David H. Edgar

## Introduction

Early detection and aggressive chemotherapy/radiotherapy treatments have improved the long-term survival rates for many young women with various types of cancer. As a consequence of these cytotoxic treatments, their reproductive future can be either short lived or eradicated. For young single women with cancer, oocyte cryopreservation offers the best potential option for achieving a future pregnancy using their own gametes. Unfortunately, the urgent need to commence cytotoxic treatment often does not permit adequate time for cryopreservation of mature oocytes. Conversely, cryopreservation of ovarian tissue eliminates the delay necessary to obtain mature oocytes, but the subsequent potential for establishing pregnancy is currently unknown. Although ovarian tissue cryopreservation is an attractive alternative and frequently used for patients with these conditions, little has been published on the efficacy of various protocols. Cryopreservation of ovarian tissue is more complex than that of gametes or embryos, requiring preservation of multiple cell types, which may vary in volume and water permeability. Essentially, ovarian tissue cryopreservation is more similar to organ cryopreservation than to that of gametes or embryos.

Early attempts to cryopreserve mouse ovarian tissue had shown the potential of this technology with evidence of intact follicles in cryopreserved tissue after grafting [1] and subsequent litters from grafts of cryopreserved tissue [2]. A resurgence of interest in ovarian tissue cryopreservation stemmed from the birth of a lamb following cryopreservation and grafting of ovarian tissue reported by Gosden *et al.* in 1994 [3]. This success has now been translated into the human with the demonstration of resumption of cycling and live births following grafting of cryopreserved ovarian tissue. However, there is little information on the relative efficiency of the methods presently being used for human ovarian tissue cryopreservation. In fact, unequivocal clinical evidence of preservation of function in autografts using current methods is limited to eight patients [4–11; C. J. Stern, personal communication], in which embryos were formed in vitro from oocytes aspirated from heterotopic grafts although no pregnancies resulted (Table 28.1 [10–11]). Despite providing proof of principle, this highlights the paucity of systematic information regarding cryopreservation of human ovarian tissue.

## Human ovarian tissue

The structure of the human ovary is a crucial consideration in the potential success of cryopreservation. The human ovarian cortex is predominantly (>80%) populated with quiescent primordial stage follicles [12–14], each consisting of an oocyte (approximately one third the diameter of a mature oocyte) surrounded by a single layer of flattened pre-granulosa cells (Figure 28.1a) or with a mixture of flattened and cuboidal pre-granulosa cells (Figure 28.1b). Follicle classification is reported in Table 28.2 [14–17] using both the B–D system reported by Gougeon and Chainy in 1987 [15] or by name [14, 17]. A much lower proportion of follicles will have initiated development (primary follicle; Figure 28.1c) and in approximately 3% of follicles division of granulosa cells will have occurred (proliferating follicle; Figure 28.1d). In contrast to the rodent ovary, in which much of the initial ovarian tissue cryopreservation was performed, more advanced follicles characterized by at least two layers of granulosa cells (secondary follicle; Figure 28.1e) and follicles with small

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**Table 28.1** Cryopreservation methodology used in clinical cases where oocytes and embryos have been derived from heterotopic grafts

| Size of tissue slices (mm) | Cryoprotectants | Dehydration | Start temp. | Seed temp. | Thaw temp. | First-thaw solution | Outcome | Ref. |
|---|---|---|---|---|---|---|---|---|
| 5 × 5 × 1 and 15 × 5 × 2 | 1.5 M DMSO +0.1 M sucrose | 4°C 30 min | 0°C | –7°C | Air 30 s then 37°C 2 min | 1.5 M DMSO + 0.1 Msucrose | 20 oocytes 8 × MII 2 × 2 PN 1 × 3 cell 1 × 4 cell ET | Oktay *et al.* [4] |
| 5 × 5 × 1 | 1.5 M EG +0.1 M sucrose | 4°C 30 min | NP | –9°C | 37°C | 0.75 M EG + 0.25 M sucrose | 3 oocytes 2 × MII 1 × 4 cell ET 1 × 5 cell ET | Rosendahl *et al.* [6] Schmidt *et al.* [10] |
| 5 × 5 × 1 and 10 × 10 × 1 | 1.5 M DMSO +0.1 M sucrose | 4°C 30 min | NP | –7°C | 35°C 2–3 min | 1.0 MDMSO + 0.1 M sucrose | 6 oocytes 4 × 2 PN 1 × 6 cell 1 × 3 cell 1 × 2 cell (no ET) | Kim *et al.* [8] |
| 5 × 5 × 2 | 1.5 M DMSO +0.1 M sucrose | 4°C 30 min | 4°C | –7°C | Air 2 min then 25°C 2 min | 1.5 M DMSO | 2 oocytes 1 × 3 cell ET | Demeestere *et al.* [7] |
| 10 × 10 × 1 | 1.5 M DMSO +0.2 M sucrose | 4°C 30 min | 4°C | –9°C | Air 30 s then 37°C 2 min | | 20 oocytes 6 MII 1 × 7 cell (no ET) | Poirot *et al.* [5] Poirot *et al.* [11] |
| NP | 1.5 M DMSO +0.1 M sucrose | 4°C 30 min | 4°C | –7°C | Air 30 s then 37°C 2 min | 1.4 M DMSO + 0.2 M sucrose | 10 oocytes 9 embryos 4 blastocysts 2 blastocysts ET | Piver *et al.* [9] |
| 3 × 2 × 1 | 1.5 M PROH +0.1 M sucrose | 22°C 30 or 90 min | 22°C | –7°C | 37°C 2 min | 1.0 M PROH +0.2 M sucrose | 14 oocytes 10 MII 2 × 2 cell ET 2 × 6 cell ET | C. J. Stern, personal communication |

All cryopreserved using controlled rate slow freezing at 2°C/min to seed temperature followed by 0.3°C/min to –40°C or –50°C.
DMSO, dimethyl sulfoxide; EG, ethylene glycol; ET, embryo transfer; MII, metaphase-II oocyte; NP, not provided; PN, pronuclei; PROH, propanediol.

**Table 28.2** Follicle classification

| Type[a] | Name[b] | Characteristic | Follicle diameter (μm) | Population (%) |
|---|---|---|---|---|
| B | Primordial | A layer of flattened pre-granulosa cells | 35–40 | 41–56 |
| B/C | Transitory/ intermediary primordial | Mixture of flattened and cuboidal pre-granulosa cells | 37–44 | 41–22 |
| C | Primary | A single layer of cuboidal granulosa cells | 46–54 | 15–21 |
| C/D | Proliferating | An incomplete second layer of granulosa cells | 60–77 | 1–3 |
| D | Secondary | Two or more layers of granulosa cells | 100 | |
| – | Antral | Multiple layers of granulosa cells with antral cavity | – | <1 |

[a] Gougeon and Chainy [15]; Gougeon [16].
[b] Westergaard *et al.* [14]; Gook *et al.* [17].

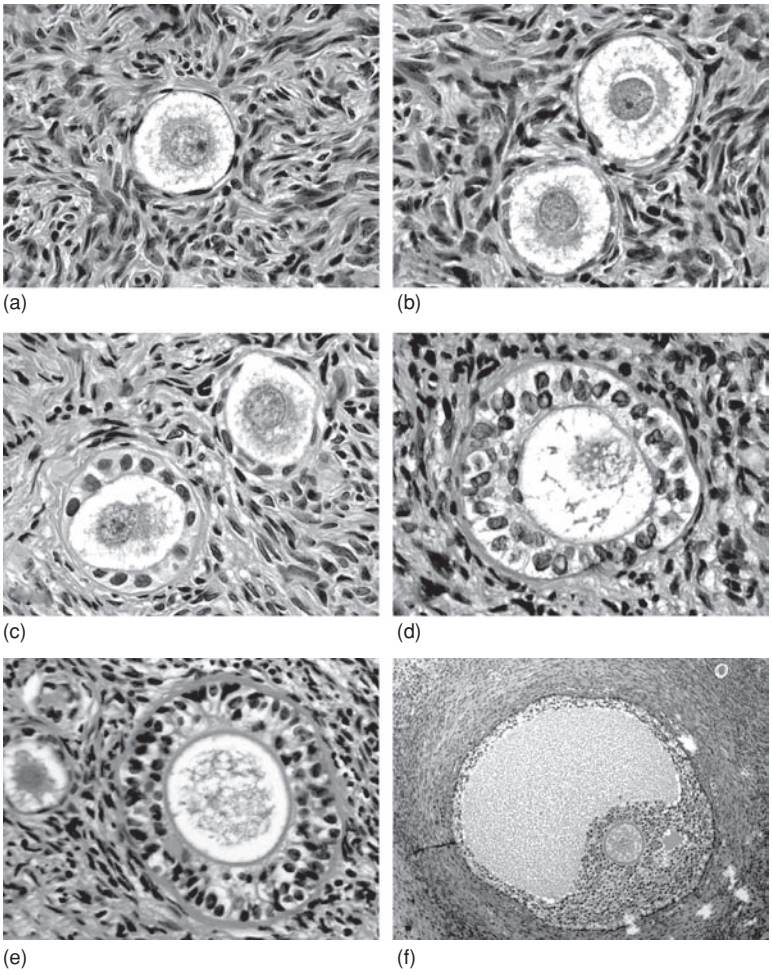



**Figure 28.1** Types of follicles observed in human ovarian tissue: (a) primordial; (b) two intermediary primordial; (c) an intermediary primordial (top) and primary (bottom); (d) proliferating; (e) secondary and an intermediary primordial; (f) an antral and a primordial (top right corner). See plate section for color version.

antral cavities (Figure 28.1f) are rarely observed in the human ovary.

Primordial follicles in the human are situated approximately 1 mm below the cortical epithelium (Figure 28.2a) embedded in a dense cortex of stromal cells and collagen bundles. At birth, it is estimated that between 250 000 and 500 000 primordial follicles [12] are present within the human ovary, decreasing

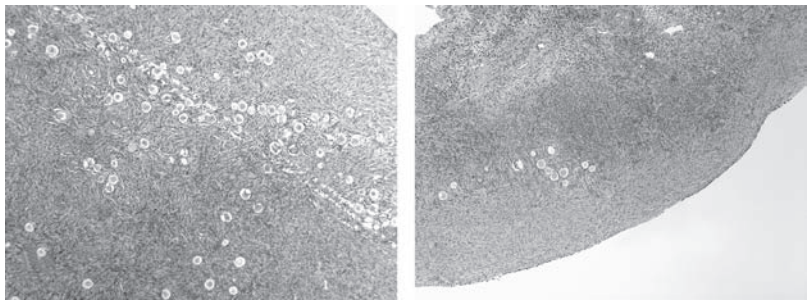



**Figure 28.2** Ovarian cortex from 2 patients: (a) an 18-year-old patient with abundant primordial follicles throughout the cortex; and (b) a 34-year-old patient with a cluster of primordial follicles. See plate section for color version.

to approximately 25 000 by the age of 37 [18]. Obviously these are the candidate follicles to preserve with any cryopreservation regimen. The aim of cryopreservation is not only to preserve the structure and function of both the pre-granulosa cells and oocyte within individual primordial follicles but also to preserve the majority of the primordial follicles. This is complicated by the enormous variation in the density of the ovarian cortex and the distribution of follicles between patients. Histological examination of ovarian tissue destined for cryopreservation from over 150 patients, ranging in age from the early teens to over 40 years of age [D. A. Gook, unpublished observations] shows that the cortex of the adult human ovary is extremely fibrous with highly variable distribution of follicles [19]. In the young teenage ovary, abundant follicles appear to be evenly distributed around the cortex (Figure 28.2a). However, in ovaries from women of more advanced reproductive age, follicles are depleted from specific regions, altering the appearance to that of follicle clusters with fewer individual follicles which are, in turn, becoming more sparsely distributed (Figure 28.2b). This lack of uniformity across the cortex will impact not only on the evaluation of methodology but also the potential clinical success in older women requesting ovarian tissue cryopreservation.

# Methodology

## Preparation of ovarian tissue for cryopreservation

The most significant loss of follicles results not from cryopreservation but from ischemia; 65% of sheep follicles are lost following exposure to 0°C for 2–3 h in a cell culture medium (Leibovitz L-15) and only a further 7% as a result of cryopreservation [20]. Under the same conditions, a slightly higher loss due to ischemia (78%) was observed in mouse ovaries with no further reduction as a consequence of cryopreservation [21]. Despite its critical impact, the preparation of ovarian tissue has received little attention. This is probably a consequence of the apparent normality of tissue as assessed by routine histological assessment of non-frozen tissue fixed at the end of preparation [D. A. Gook, unpublished observations] and the high level of viability staining after thawing [22]. This fails to detect the consequences of ischemic exposure during preparation, which only becomes evident when the tissue is grafted and begins to function.

It is routine practice for organs destined for transplantation to be perfused with and transported for a number of hours in a basic salt solution at 4°C in order to reduce ischemia. However, it is questionable whether this rationale would apply to ovarian tissue in which the follicles are located just under the surface epithelium where there is minimal circulation.

Collection and preparation of ovarian tissue is most commonly carried out in Leibovitz L-15 medium at 4°C. Due to the limited number of centers performing ovarian tissue cryopreservation, tissue is frequently transported for 4–5 h [23] and has been transported in some cases for up to 28 h [24]. The consequences of these conditions are largely unknown. A reduction in both primordial (60%) and developing follicles (40%) has been observed following exposure of intact rat ovaries to 4°C for 24 h prior to subsequent transplantation [25].

It is possible that cold ischemia may be reduced by transport in a more appropriate medium. A comparison of exposure of human ovarian tissue to 4°C for 24 h in a Leibovitz-based medium or a histidine–tryptophan–ketoglutarade solution (HTK, an organ transport medium) showed enhanced follicle survival and a lower level of lipid peroxidation in the HTK

[26]. The HTK medium is currently used for extended duration transport of ovarian tissue [26]. However, a similar comparison of a culture medium designed for gametes and embryos (Quinns Hepes modified HTF; Figure 28.3a) and HTK (Figure 28.3b) for a shorter duration (2 h) followed by xenografting resulted in a dramatic loss of stromal tissue structure and follicles regardless of the medium used [D. A. Gook, unpublished observations].

Whether ambient temperature would be more appropriate for transport is difficult to ascertain. Similar levels of ischemia (measured by the rate of oxygen consumption) were observed in bovine ovaries following 24 and 48 h incubation at 4°C compared to ambient temperature [27]. In animal studies, it is clear that exposure of antral follicles in a whole ovary to 4°C results in impaired developmental potential in the oocytes following fertilization [28], but information relating to the impact on primordial follicles is scarce. In our study, less ischemic damage was observed following exposure of human ovarian tissue to ambient temperature (22°C) for 2 h (Figure 28.3c) than cold exposure. However, both warm and cold ischemia result in loss of cell structure and a reduction in the volume of ovarian tissue, with the damage reaching a maximum at 4 days, generally when it appears that neovascularization has occurred [29]. In contrast to this study, bovine ovaries exposed for 3 h to cold or warm conditions showed no increase in ischemia (as measured by oxygen consumption) irrespective of treatment [27]. A reduction in ischemic damage and increased follicle survival has been reported following daily dietary supplementation of vitamin E in mice after ovarian grafting [30].

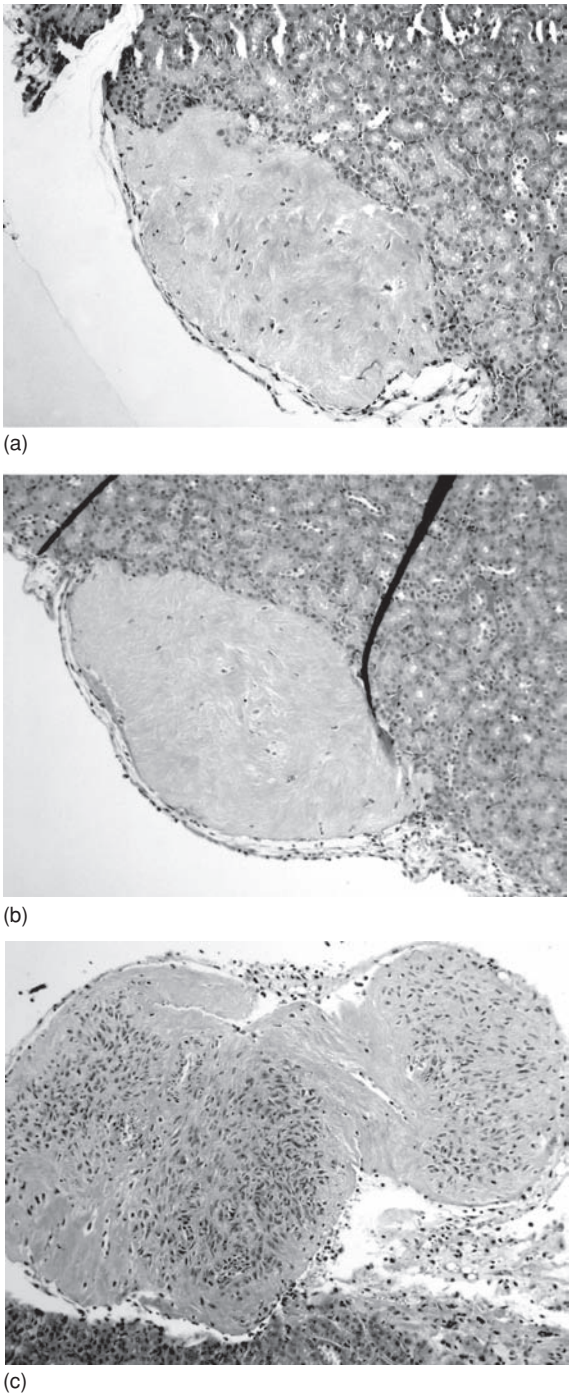



**Figure 28.3** Fresh ovarian tissue exposed to different temperatures for 2 h prior to xenografting under the kidney capsule in immunodeficient mice and subsequently examined 2 days after grafting. (a) Tissue in Quinns Hepes modified human tubal fluid (HTF) at 4°C. (b) Histidine–tryptophan–ketoglutarade solution (HTK) medium at 4°C. (c) Quinns Hepes modified HTF at 22°C. (a) and (b) magnification ×10, (c) ×5. See plate section for color version.

## Tissue dissection

Obviously, incubation at 37°C in an in vitro fertilization (IVF) medium is also beneficial for the collection of oocyte cumulus complexes (OCC) from antral follicles during tissue preparation. Generally, two types of OCC are recovered: those with very few layers of corona cells, frequently with corona cells in the perivitelline space (an indicator of atresia); and those with a dense compact mass of corona cells in which only a vague outline of the oocyte can be seen. Our experience suggests that only a minor proportion of these oocytes will mature in culture and this may take as long as 72 h, at which stage they can be denuded and cryopreserved. In 2004, Isachenko

*et al*. reported two cases where oocytes were recovered during ovarian tissue preparation with 50% maturing after 36 h in culture in an in vitro maturation (IVM) culture system [31]. In some patients with endometrial cancers there is an associated predisposition to polycystic ovaries and priming with human chorionic gonadotropin (hCG) prior to ovarian harvest [32] may be beneficial in these situations, resulting in a higher proportion of mature oocytes [33]. Follicles can also be collected from human ovarian tissue following enzyme and mechanical isolation [34–36]. Although these follicles can potentially be cryopreserved, the efficiency of this has yet to be accurately assessed in the human. However, isolated murine follicles have been successfully cryopreserved using both slow freezing [37–40] and, more recently, using vitrification [41–43].

Attempts have been made to cryopreserve the intact ovary (see Chapter 30). However, due to the extremely fibrous structure of the human ovarian cortex and the location of the primordial follicles, the general consensus approach has been to minimize the size of the tissue pieces. Human ovarian tissue is prepared by first removing all medulla and reducing the cortical thickness to approximately 1 mm. This facilitates cryoprotectant and water movement with the ultimate aim of balancing effective dehydration with minimal injury due to osmotic toxicity. To increase surface area exposure to cryoprotectants, and facilitate storage, the 1 mm thick cortical tissue is further sliced into pieces ranging in surface area from 1–15 $mm^2$ [44]. There is, however, no clear evidence that can be used to establish a relationship between the dimensions of a tissue slice and the successful preservation of follicles.

## Cryopreservation

In contrast to cryopreservation of gametes and embryos, ovarian tissue cryopreservation is more closely related to organ cryopreservation [45]. The underlying problem is that the optimal cryopreservation procedure for one cell type may not be optimal for other cells within the tissue. Therefore, a compromise may be necessary to preserve a high proportion of all cell types present in the tissue. There is obviously disparity of cell size between the stromal cells, the oocyte and the pre-granulosa cells within the primordial follicle, which will limit the ability to optimize the cryopreservation procedure for human ovarian tissue. The rate at which water traverses the cell membrane, the membrane hydraulic permeability (Lp) [46], is a fundamental consideration in cryopreservation and will be specific for a cell type. In some cases, such as the human oocyte, the Lp varies between cells of the same type [47]. This cellular property, together with the surface area and free water content of a cell, will dictate the rate of dehydration necessary prior to cryopreservation regardless of whether cryopreservation is achieved via controlled rate cooling or vitrification. In addition, cell size, which will contribute to determining the optimal rate of cooling [45], is not uniform within ovarian tissue.

### Cryoprotectants

Cellular density and tissue geometry will affect infiltration of cryoprotectants. Diffusion of cryoprotectants is relatively rapid in murine ovaries, which consist almost completely of abutting developing follicles with almost no fibrotic material and are therefore relatively porous. However, the densely fibrotic human ovarian cortex is extremely different. Murine ovarian tissue is therefore an unsuitable model for the human in this respect.

In the case of permeating cryoprotectants, the aim is to gradually displace cellular water without inducing excessive shrinkage. To overcome the difficulties associated with the density of human ovarian tissue, the use of higher concentrations of cryoprotectants has been suggested. However, under these circumstances, the reduced aqueous phase and hyperosmotic conditions result in excessive shrinkage of cells and loss of cell–cell communication [48]. This, in turn may result in subsequent impairment of tissue function even though morphology may appear normal. The reduction in filamentous actin traversing the zona of oocyte cumulus complexes isolated from cryopreserved murine ovaries indicates that this may occur in ovarian tissue [49].

Four permeating cryoprotectants: glycerol (GLY), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propanediol (propylene glycol; PROH) have been used in human and animal ovarian tissue cryopreservation. For all permeating cryoprotectants, a concentration of 1.4 or 1.5 mol/l has generally been used for slices of human and animal ovarian tissue [44]. However, higher concentrations of PROH (2 and 4 mol/l at ambient temperature) do not appear to cause follicular toxicity when compared to control tissue exposed

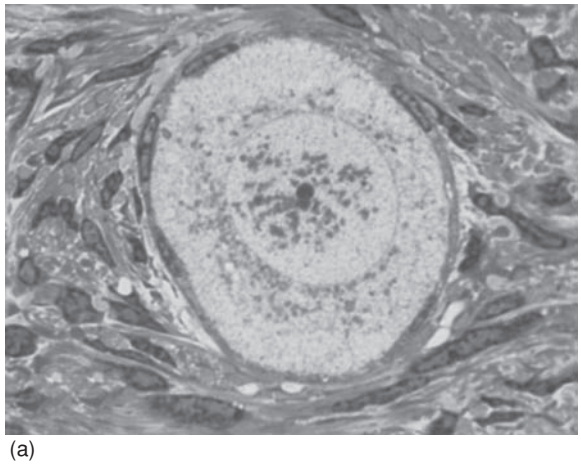
to no cryoprotectant [50]. In contrast, concentrations of DMSO above 2 mol/l were toxic. Follicular toxicity has also been shown for concentrations of EG above 2.0 mol/l [51].

Although GLY was used in initial rodent studies [1, 2, 52], it has not been used clinically for human tissue. This is probably due to the reported low proportion of follicles (10%) surviving after cryopreservation of human tissue with glycerol, the subsequent lack of follicles observed in the majority of grafts using this tissue [53] and the failure of follicles to survive in vitro culture [54]. The poor outcome observed with glycerol is likely to be a consequence of the slow rate at which glycerol permeates tissue relative to other cryoprotectants [55], although this can be compensated for by increasing the dehydration time [56].

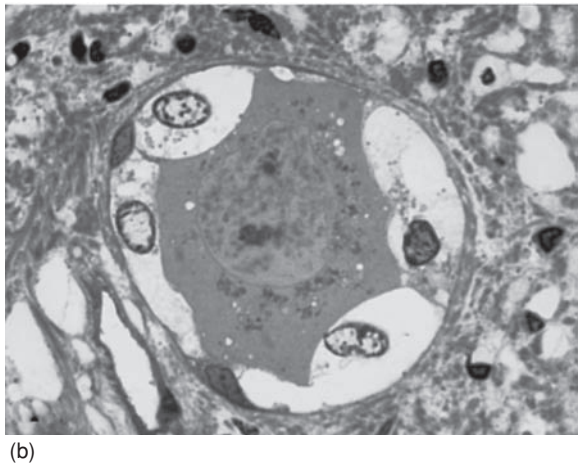
The rate of cryoprotectant penetration through tissue is also a function of temperature. Newton *et al.* showed that both DMSO and EG had penetrated through 76% of human ovarian tissue in 20 min at 4°C but that this occurred twice as rapidly at 37°C [55]. Of interest is the additional observation that neither of these cryoprotectants had permeated all of the tissue even after 90 min at 37°C. In contrast, PROH penetration was slower at 4°C than DMSO and EG, requiring 30 min to achieve 76% penetration but at 37°C PROH had a significantly higher rate of diffusion, achieving 100% penetration by 15 min. It is of no surprise then that reduced follicle survival was observed following dehydration of larger pieces of human ovarian tissue at 4°C for 30 min in PROH (44%) relative to DMSO (84%) and EG (74%) [53]. High follicle survival following cryopreservation under the same conditions using EG has also been observed by others [57]. Similar levels of survival (>80%) have been achieved using appropriate conditions for individual cryoprotectants i.e DMSO at 4°C and PROH at room temperature [58]. However, to achieve a similar survival rate using a short (10 min) exposure time with larger (2 × 10 × 10 mm) pieces of sheep ovarian cortex, which is also relatively fibrous, a higher concentration of either PROH or DMSO (2 mol/l) was necessary [50]. Effective dehydration of tissue is therefore dependent on the tissue geometry together with the rate of penetration of the cryoprotectant which is in turn a function of temperature. Therefore, to conclude from some of the above studies that a cryoprotectant is unsuitable for ovarian tissue cryopreservation is not justified. It would appear that insufficient dehydration may have resulted from suboptimal conditions for a specific cryoprotectant and that, with optimization, a variety of protocols may be appropriate.

Non-permeating cryoprotectants, generally sucrose at a concentration of 0.1 mol/l, have also been used to facilitate dehydration of ovarian tissue [11, 13, 58–61]. Recent studies have shown that increasing the sucrose concentration significantly improves outcomes for embryo cryopreservation [62] but addition of various concentrations of sucrose in combination with DMSO did not appear to result in improved cryopreservation [55] and no other studies have specifically compared protocols with and without sucrose.

The use of sucrose with the permeating cryoprotectant PROH [13] has been investigated in an attempt to optimize dehydration of both pre-granulosa cells and oocytes within primordial follicles [63]. Equivalent proportions of morphologically intact oocytes could be achieved with shorter time exposure when the sucrose concentration was increased from 0.1 to 0.2 mol/l. However, extended exposure to higher sucrose resulted in decreased oocyte survival [63] and the observation, within the surviving oocytes, that the majority of cytoplasm consisted of vacuolation and lysed mitochondria. This phenomena was not observed with the lower sucrose concentration (0.1 mol/l) which, in contrast, resulted in an improvement in the proportion of morphologically intact oocytes and pre-granulosa cells with time. The proportion of surviving oocytes with normal cytoplasm also increased with time in 0.1 mol/l sucrose [13]. Extended exposure to the elevated sucrose concentration may result in inappropriate osmotic gradients which is also the likely explanation for the low survival of pre-granulosa cells (Figure 28.4b) and poor cytoplasmic morphology in oocytes when applying two-step compared to single-step dehydration with equivalent final cryoprotectant concentrations [13]. By manipulation of dehydration using PROH and sucrose at ambient temperature, a high proportion of intact pre-granulosa cells (74%), intact oocytes (91%) and oocytes with normal cytoplasmic appearance (95%) could be achieved (Figure 28.4c). However, morphology of the stromal tissue was consistently poor irrespective of the regimen (Figure 28.4b,c) compared to non-cryopreserved (Figure 28.4a), highlighting the relative complexity associated with dehydration of tissue containing multiple cell types.



(a)



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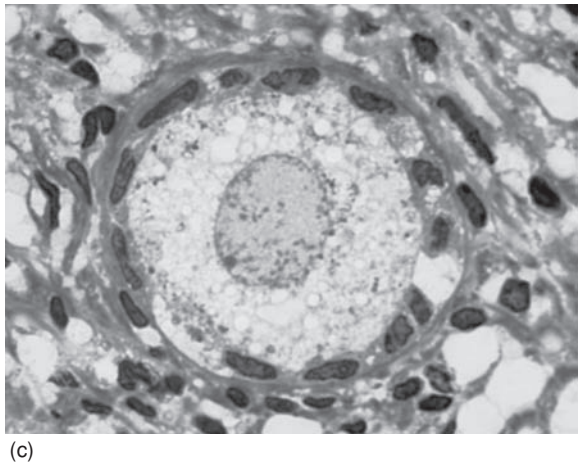


(c)

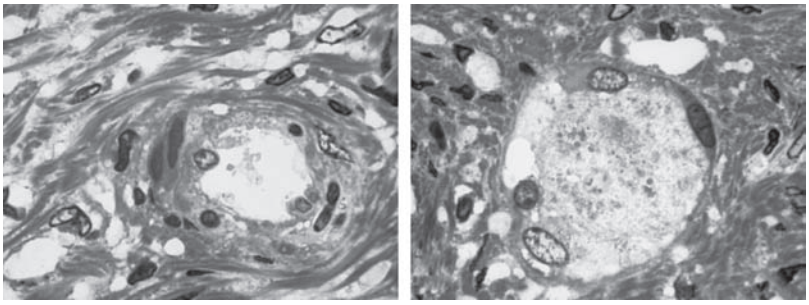
**Figure 28.4** Primordial follicles present in ovarian tissue following cryopreservation: (a) non-cryopreserved; (b) dehydrated using 1.5 M propanediol (PROH) and 0.2 M sucrose; (c) dehydrated using 1.5 M PROH and 0.1 M sucrose. See plate section for color version.

## Rate of cooling

In the majority of the above studies the rate of cooling used has been similar to the rates used for controlled rate embryo cryopreservation (2°C/min to ice seeding temperature followed by 0.3°C/min). As with embryo and oocyte cryopreservation, the rate of cooling for ovarian tissue has never been systematically optimized. Mazur estimated that the theoretical rate of cooling for organs or tissues should be <1°C/min [45], but a comparison of the standard slow rate (0.3°C/min) and a faster cooling rate (2°C/min) with large pieces (200 $mm^3$) of ovine ovarian tissue demonstrated a significant improvement in follicular survival at the faster rate [50]. In an attempt to investigate this for human ovarian tissue, the slow rate (0.3°C/min) was compared to two faster cooling rates following the same prefreeze dehydration and the morphology of each cell type was determined [13]. Poor morphology of the whole tissue was observed with a rapid rate (Figure 28.5a; to be discussed in more detail later in the context of vitrification). At an intermediate rate (~36°C/min), a high proportion of the stromal cells and collagen bundles appeared normal but only half the oocytes were intact and almost all pre-granulosa cells were abnormal (swollen and with swollen nuclei; Figure 28.5b), again emphasizing the importance of variation in cell size. With the slow rate, approximately half of both the stromal and pre-granulosa cells and over 80% of the oocytes were normal. Normal appearance in the pre-granulosa cells and oocytes could be further enhanced by applying the slow rate after more extensive dehydration prior to cryopreservation but this was at the expense of stromal cell survival (Figure 28.4c) [63, 64].

During controlled rate cryopreservation, cryoprotectant crystallization will occur, and the temperature at which this occurs is specific for the cryoprotectant. For PROH or DMSO this occurs at –6 to –8°C. Without manual seeding, crystallization will be initiated at any solid surface i.e. throughout the tissue. Demirci *et al.* [50] reported a dramatic deviation from the normal cooling curve resulting in reduced follicular survival in the absence of manual seeding. Manual seeding at a slightly higher temperature (–5 compared to –7°C) appeared to improve follicle survival [54].

Similarly, damage can occur during thawing as a result of crystallization of water or cryoprotectant. Irrespective of the cryopreservation method used (controlled rate or vitrification), thawing should be as



(a) (b)

**Figure 28.5** Ovarian tissue cryopreserved using: (a) a rapid cooling rate showing remnants of a primordial follicle; and (b) an intermediate rate of cooling showing a primordial follicle containing lysed pre-granulosa cells and oocyte. See plate section for color version.

rapid as possible. Although critical, the thawing temperature is rarely reported and, again, almost no studies have systematically investigated this aspect of cryopreservation with animal or human ovarian tissue. Exposure to some cryoprotectants, such as DMSO, at higher temperatures may increase their toxicity. A comparison of thawing of murine ovarian tissue, cryopreserved in DMSO, at 27, 37 or 47°C showed a significant reduction in follicle survival at the higher temperatures [54]. In our experience, thawing tissue cryopreserved in PROH in a 1 ml volume in a Cryovial requires 3 min at 37°C to achieve liquefaction prior to immediate removal of the tissue. At this temperature, at least in the case of PROH, there appears to be no affect on developmental potential (see later).

Vitrification offers the potential benefit of overcoming many of the issues associated with crystallization discussed above. The problem associated with vitrification of ovarian tissue, however, is how to achieve the high cooling and warming rates required. Vitrification of murine ovaries has been successful with subsequent births of pups reported [65, 66] following vitrification in cryostraws and plunging in liquid nitrogen. However, when a faster cooling rate was achieved by direct contact with liquid nitrogen, it resulted in better preservation as evidenced by significantly more morphologically normal, viable follicles and pups [65]. This improvement, however, may also be partly due to more appropriate dehydration prior to vitrification. Vitrification in cryostraws resulted in poorer outcomes relative to controlled rate cryopreservation for all parameters measured. Wang *et al.* [67] have also shown the importance of the cooling rate for vitrification with both mouse and human ovarian tissue.

Ovine hemi-ovaries have also been successfully vitrified resulting in live births although one of the four births was a large lamb and died short after delivery as a result of malformations [68]. The cocktail of cryoprotectants (2.6 M DMSO, 2.6 M acetamide, 1.3 M PROH and 7.5 mM polyethylene glycol) used in this study has also been used for human ovarian tissue with evidence of preservation of normal follicular morphology [69]. In contrast, poor developmental capacity was observed following vitrification of murine embryos with this protocol [70].

It is of some concern that, in order to achieve dehydration of ovarian tissue and facilitate vitrification with rapid instead of ultra-rapid rates of cooling, exposure to very high concentrations of a cocktail of cryoprotectants may be necessary. In contrast to embryo and oocyte vitrification, in which exposure to these very high concentrations of cryoprotectants is limited to <30 s and many normal births have been reported, ovarian tissue may be exposed for 15 min. Dehydration prior to controlled rate cryopreservation in DMSO (1.5 M) has routinely been performed at 4°C to limit toxicity [71]. Using this approach, successfully vitrification of human ovarian tissue has been achieved with dehydration in 1.5 M DMSO at 4°C in combination with PROH and EG [72]. However, in a another recent vitrification procedure, human ovarian tissue was exposed to 1.0 M DMSO followed by 2.8 M DMSO at ambient temperature for 15 min at each concentration [73]. The safety of the vitrification procedures required for ovarian tissue will require careful evaluation in animal models before clinical application.

## Storage

Irrespective of the methodology used for cryopreservation, tissue has generally been stored in Cryovials, which do not constitute a fully sealed system. A

theoretical risk of cross contamination is associated with storage of biological material in Cryovials under liquid nitrogen and the relatively extended duration of storage which may be anticipated with ovarian tissue would potentially increase this risk. As such, storage in liquid nitrogen vapor may be the preferred option for facilities offering clinical ovarian tissue cryopreservation.

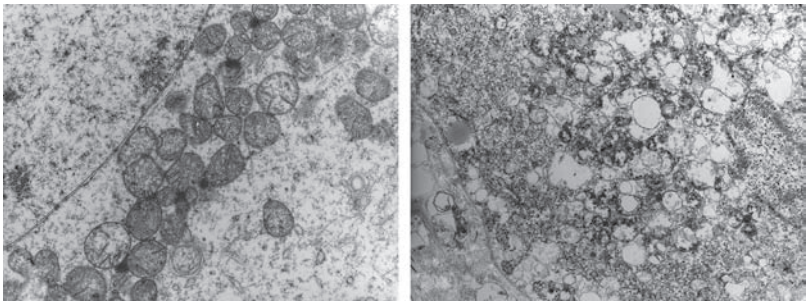
## Evaluating the efficiency of ovarian tissue cryopreservation

In contrast to evaluation of outcomes from embryo or gamete cryopreservation, assessing the survival and viability of cryopreserved ovarian tissue poses specific challenges. In many of the studies discussed previously, success has been measured in terms of follicle survival after isolation from thawed tissue. The validity of this approach depends on three fundamental assumptions: (a) that lysed or destroyed follicles will be detected after isolation; (b) that loss of follicles as a result of enzyme digestion will be equivalent for cryopreserved and fresh tissue; and (c), specifically in the case of human ovarian tissue, that there is an even distribution of follicles throughout the tissue. However, once an oocyte within a primordial follicle has lysed, it is essentially impossible to identify the follicle (Figure 28.5a,b). Therefore, only follicles with an intact oocyte will be included in these studies, resulting in over estimation of survival. In addition, much of the stromal tissue is damaged with controlled rate cryopreservation rendering follicles within it more vulnerable to enzymes and this is also likely to vary between protocols (Figures 28.4 and 28.5). Finally, due to the extent of variation in follicle distribution in human ovarian tissue, it is invalid to express the number of follicles present after cryopreservation as a proportion of the number in a non-cryopreserved sample. Although these criticisms weaken some of the conclusions which have been drawn above there are no other available studies of this type on cryopreservation of ovarian tissue.

In contrast, it is potentially possible to overcome these problems by histological evaluation of the entire tissue but there are very few studies which have attempted this [21] and expressed normal morphology as a proportion of the total number of follicles within a piece of tissue [13, 63, 74]. Histological examination, at both the light and electron microscope level, has generally assessed only a small sample to estimate overall follicle integrity [75], although this has allowed detection of abnormalities such as oocyte shrinkage [76], vacuolated areas within the oocyte cytoplasm [77], loss of mitochondrial cristae [78] and lysis of pre-granulosa cells [79].

There are only two morphometric studies of human ovarian tissue which assess cryopreservation [13, 74], one of which is an evaluation of the most commonly used procedure [3] using DMSO as a cryoprotectant and controlled rate cooling on tissue from six patients [74]. In this study, almost half of the follicles and the vast majority of oocytes (81%) were damaged. Parallel assessment of apoptosis confirmed these observations. The other study assessed the proportion of intact pre-granulosa cells and oocytes together with the relative normality of the oocyte cytoplasm as estimated by vacoulation and normal mitochondria within every oocyte for a range of cryopreservation procedures using PROH and sucrose as cryoprotectants [13, 63]. Observations were verified by electron microscopic evaluation of a small number of follicles (Figure 28.6a,b). The highest proportion of both oocytes (85%) and pre-granulosa cells (74%) with normal morphology was observed following dehydration for 90 min in 1.5 M PROH + 0.1 M sucrose at ambient temperature followed by a slow controlled rate of freezing. Although this type of morphometric assessment has provided evidence of morphological normality after cryopreservation, it gives no indication of viability or function.

Viability staining has also been used to assess follicles within a piece of tissue [80, 81] following cryopreservation. Generally, this staining will identify live cells on the basis of an intact membrane and cytoplasm which is functionally capable of cleaving a chromagen, and dead cells on the basis of their inability to exclude a nuclear stain [51, 57]. Again, this form of assessment has limitations [76]. Tissue pieces must be very small to facilitate diffusion of dye and only follicles with a live oocyte are detected. Although the nuclear stain will detect the germinal vesicles (GV) of lysed oocytes, these are indistinguishable from the nuclei of stromal or pre-granulosa cells, resulting in the potential for overestimation of follicle viability. An example of a lysed oocyte which would be unlikely to be distinguished from a granulosa cell is shown in Figure 28.5a. Finally, it is important to remember that we cannot infer, on the basis of viability staining, that the developmental potential of these primordial follicles has been retained following cryopreservation.



(a) (b)

**Figure 28.6** Electron micrographs of oocyte cytoplasm within primordial follicles after cryopreservation using: (a) dehydration in 1.5 M propanediol (PROH) and 0.1 M sucrose for 90 min followed by slow cooling showing normal mitochondria and cytoplasm; and (b) following same regimen with a shorter time of 30 min showing loss of mitochondria, vacuolation and abnormal cytoplasm.

## Developmental potential

Assessment of expression of developmental potential in vitro is an attractive possibility, but requires an understanding of the requirements for initiation of growth in primordial follicles. Additionally, many months of culture may be required. More advanced follicles isolated at the secondary stage, in which initiation of granulosa cell proliferation has occurred, can be successfully grown in culture [82–84]. However, the predominant follicle present in the human ovary is the primordial. There is evidence, that murine primordial follicles, grown in a two-phase culture system, can produce live pups [85, 86], but this has not been established using cryopreserved ovarian tissue. The culture of isolated primordial follicles, whether from fresh or cryopreserved tissue, has proved problematic in animal models [87–89] and in human [59, 90, 91]. However, follicular development to the secondary stage has been established by culturing primordial follicles within stromal tissue [92, 93]. More recently, development from primordial to early antral stage in vitro has been demonstrated using a two-stage culture system with fresh tissue [35], and there is a preliminary report of this approach being used to demonstrate developmental potential of human primordial follicles following vitrification [E. E. Telfer, unpublished data]. Results to date on demonstration of developmental potential using in-vitro systems, while promising, are limited to relatively few follicles.

As a result of the difficulties associated with culture of primordial follicles in vitro, various in vivo approaches involving grafting of cryopreserved ovarian tissue, either alone or in combination with final maturation in vitro, have been applied. Clear evidence of preservation of developmental potential has been established by the birth of live offspring from primordial follicles following grafting of ovine and murine cryopreserved ovarian tissue [3, 71, 94]. Heterotopic grafting of murine cryopreserved tissue with subsequent IVM has also resulted in live births [95].

In the human situation, xenografting of cryopreserved tissue into immunodeficient mice has been used to assess preservation of developmental potential in a number of studies and has established that follicles are viable and capable of development [21, 60, 96–98]. Developmental capacity to the antral stage has been shown to be preserved with the commonly used cryopreservation regimen using DMSO [97, 98] and also the PROH/ sucrose procedure [96]. Although primary and secondary follicles within human ovarian tissue may have survived cryopreservation, the time required for these antral follicles to develop post-grafting (5–6 months) suggests that they have developed from primordial follicles. Reproducibility of preservation has been established by development of numerous antral follicles using tissue cryopreserved from multiple patients with both procedures [99–101]. Full developmental competence has been shown to be preserved with both procedures with evidence of ovulation (corpora lutea) [98, 102] and mature oocytes [99, 100, 102]. Unfortunately, although mature oocytes were aspirated from follicles in our laboratory, experiments to determine subsequent fertilization were prohibited by law in Australia.

Clinical evidence has established that fertilization and embryo development can occur in oocytes recovered from cryopreserved ovarian tissue transplanted at a heterotopic site [4–11; C. J. Stern, personal communication], but no pregnancy has resulted following transfer of these embryos to date. The cryopreservation procedures which have been shown to be associated with preservation of full developmental potential are reported in Table 28.1. At present, consistent follicle development and recovery of mature oocytes has proved difficult.

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Chapter 29

# Ovarian tissue transplantation

Jacques Donnez, Jean Squifflet and Marie-Madeleine Dolmans

## Introduction

According to previous reports, around 700 000 new cancer cases are expected every year among the female population of the USA [1] and 8% of these women are likely to be under the age of 40. In fact, it is now estimated that 1 in every 250 people in the adult population is a childhood cancer survivor [2].

Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly increased the life expectancy of premenopausal women with the disease. Indeed, aggressive chemotherapy and radiotherapy, as well as bone marrow transplantation, can cure more than 90% of girls affected by childhood malignancies [3], but have resulted in a growing population of adolescent and adult long-term survivors of childhood cancer [2] who may experience infertility problems due to induced premature ovarian failure (POF).

## Indications for ovarian tissue cryopreservation

Oncological indications for ovarian tissue cryopreservation are summarized in Table 29.1 [4, 5]. In case of gynecological malignancy, a conservative fertility approach is only valuable if the uterus can be spared during surgery. This includes cases of early cervical carcinoma, early vaginal carcinoma, early endometrial adenocarcinoma, ovarian tumors of low malignancy and some selected cases of unilateral ovarian carcinoma (stage IA) [4, 5]. The choice of a possible conservative surgical approach in these patients and the question of implementing such treatment alone remain controversial, and all published results were obtained on the basis of retrospective studies and/or case reports. The fertility outcome is conditioned by the adjuvant therapy administered, i.e. local radiotherapy and/or chemotherapy. Respective indications in the case of malignant disease are presented in Table 29.1.

However, cryopreservation should not be reserved solely for women with malignant disease [6]. Indeed, hematopoietic stem cell transplantation (HSCT) has been increasingly used in recent decades for non-cancerous diseases, such as benign hematological disease (sickle cell anemia, thalassemia major and aplastic anemia) and autoimmune diseases previously unresponsive to immunosuppressive therapy (systemic lupus erythematosus, autoimmune thrombocytopenia) [6, 7–10]. Other benign diseases, such as recurrent ovarian endometriosis or recurrent ovarian mucinous cysts, are also indications for ovarian cryopreservation. Patients undergoing oophorectomy for prophylaxis may potentially benefit from ovarian cryopreservation too.

## Autotransplantation of cryopreserved human ovarian tissue

There have been numerous reported cases of autotransplantation of cryopreserved ovarian tissue, either to an orthotopic or heterotopic site [6, 9–24].

### Orthotopic autotransplantation of cryopreserved human ovarian tissue

In theory, natural pregnancy may be achieved via orthotopic tissue transplantation if the fallopian tubes remain intact.

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**Table 29.1** Indications for cryopreservation of ovarian tissue in cases of malignant and non-malignant disease.

| |
|---|
| **A. Malignant** |
| ***(a) Extrapelvic diseases*** |
| Bone cancer (osteosarcoma, Ewing's sarcoma) |
| Breast cancer |
| Melanoma |
| Neuroblastoma |
| Bowel malignancy |
| ***(b) Pelvic diseases*** |
| Non-gynecological malignancies |
| Pelvic sarcoma |
| Rhabdomyosarcoma |
| Sacral tumors |
| Rectosigmoid tumors |
| Gynecological malignancies |
| Early cervical carcinoma |
| Early vaginal carcinoma |
| Early vulvar carcinoma |
| Selected cases of ovarian carcinoma (stage IA) |
| Borderline ovarian tumors |
| Systemic diseases |
| Hodgkin's disease |
| Non-Hodgkin's lymphoma |
| Leukemia |
| Medulloblastoma |
| **B. Non-malignant** |
| ***(a) Uni/bilateral oophorectomy*** |
| Benign ovarian tumors |
| Severe and recurrent endometriosis |
| BRCA-1 or BRCA-2 mutation carriers |
| ***(b) Risk of premature menopause*** |
| Turner's syndrome |
| Family history |
| Benign diseases requiring chemotherapy: autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, Behçet's disease, Wegener's granulomatosis) |
| ***(c) Bone marrow transplantation*** |
| Benign hematological diseases: sickle cell anemia, thalassemia major, aplastic anemia |
| Autoimmune diseases unresponsive to immunosuppressive therapy |

From Donnez *et al.* [5]

# Technique

## Ovarian biopsy and freezing

Ovarian tissue cryopreservation is undertaken before chemotherapy. Written informed consent is obtained from the patient or her parents if she is under 18 years of age. It is always considered an emergency and ovarian biopsy is performed as soon as possible in order not to delay the start of chemotherapy.

As follicles are located inside the ovarian cortex, tissue samples collected for cryopreservation have to come from the surface of the organ. The biopsy can be taken during any gynecological procedure, by laparoscopy or laparotomy, and may be composed of one or several cortical fragments, or even a whole ovary, depending on the surgical indications and the risk of POF after treatment. Biopsies may be obtained by simple laparoscopy carried out under general anesthesia. Biopsy forceps [25] are inserted through one of the 5-mm trocars placed in the iliac fossa and are used to grasp the ovary and cut a fragment from its surface (Figure 29.1). The cortical biopsy can also be easily performed with laparoscopic scissors.

It is important to remove only the cortical surface to a depth of 1–2 mm to be sure to obtain tissue from the area rich in primordial follicles.

The number of biopsies taken varies according to the size of the patient's ovaries and the estimated risk of POF. Indeed, POF after chemotherapy is dependent on age, drug used and dose given [26]. Biopsy samples are immediately transferred to the laboratory in Leibovitz L-15 medium supplemented with Glutamax$^{TM}$ (Invitrogen, Paisley, UK) on ice. To minimize any tissue damage due to ischemia, the samples are transferred within the shortest possible interval (minutes) to the laboratory for processing. Unilateral (left) oophorectomy is only performed where there is a 100% risk of ovarian failure, like after pelvic radiotherapy, bone marrow transplantation or chemotherapy with 2 alkylating agents.

## Slow freezing procedure

The whole procedure is performed on a laminar airflux table using sterile disposable materials to ensure optimal sterility of the tissue fragments. Samples are transferred to a Petri dish containing a sterilized glass slide and 1–2 ml of Leibovitz L-15 medium. Tissue temperature is kept close to 4°C by placing the dish on top of a glass box containing crushed ice. The ovarian medulla is then separated from the cortex using forceps and surgical scissors, and disposed of. The remaining cortex is cut on the glass slide into strips of 2 × 6–8 mm. These strips are transferred into 2 ml Cryovials (Simport, Quebec, Canada) containing 800 µl of L-15 medium and stored at 0°C in a cooler

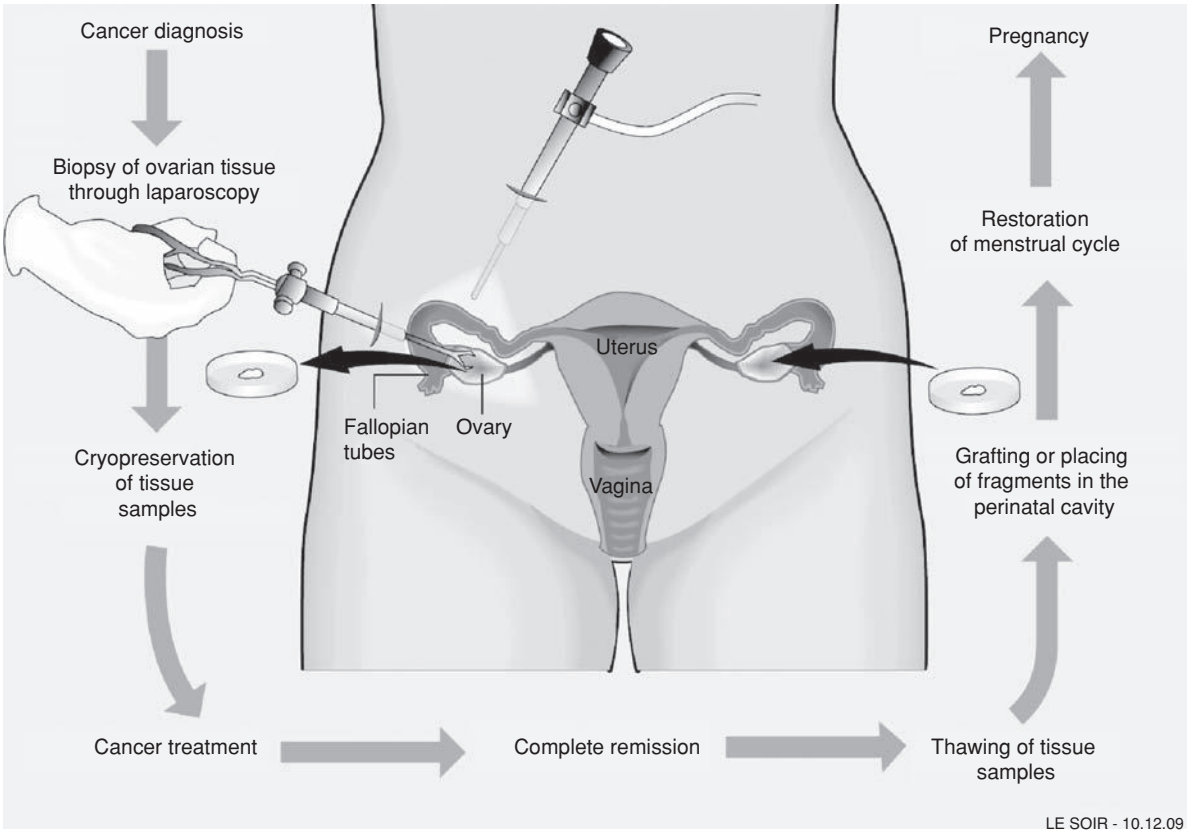



**Figure 29.1** Illustration describing the different steps from biopsy to re-implantation and pregnancy. See plate section for color version.

box (Nalgene™ Labtop cooler, Cat. No. 5116–0032; Nalge Nunc International, Rochester, NY, USA), each of the tubes containing 2–5 strips. One strip is randomly put aside and immersed in a 37% paraformaldehyde solution for histological examination and evaluation of the ovarian reserve. Leibovitz L-15 medium is then twice replaced with 800 μl of freshly prepared cryoprotective solution containing 88% Leibovitz L-15 medium, 2% human albumin 20% (Red Cross, Brussels, Belgium) and 10% dimethyl sulfoxide (Sigma-Aldrich Co., Irvine, UK).

Freezing of ovarian tissue is carried out according to the protocol described by Gosden *et al.* [27]. The Cryovials are cooled in a programmable freezer (Kryo 10, Series III; Planer, Sunbury-on-Thames, UK) following the steps below:

- At 0°C, place Cryovials inside the freezer and keep stable at 0°C for 15 min.
- From 0 to –8°C, cool at a rate of –2°C/min.
- Keep stable at –8°C for 8 min for soaking.
- Seed manually (induction of ice crystal formation) by grasping the Cryovials (for 5–10 s each) with forceps prechilled in liquid nitrogen.
- Keep stable at –8°C for 15 min.
- From –8 to –40°C, cool at a rate of –0.3°C/min.
- From –40 to –150°C, cool at a rate of –30°C/min.

The Cryovials are then transferred to liquid nitrogen (–196°C) for storage.

### Thawing [16]

For thawing, the Cryovials are removed from the liquid nitrogen and left at room temperature for 2 min. Thawing is subsequently completed by immersing the Cryovials in a warm (37°C) water bath for 2 min. The tissue samples are then grasped with small forceps and placed in a Petri dish containing L-15 medium, which is replaced 3 times (5 min each wash) to remove the cryoprotectant completely.

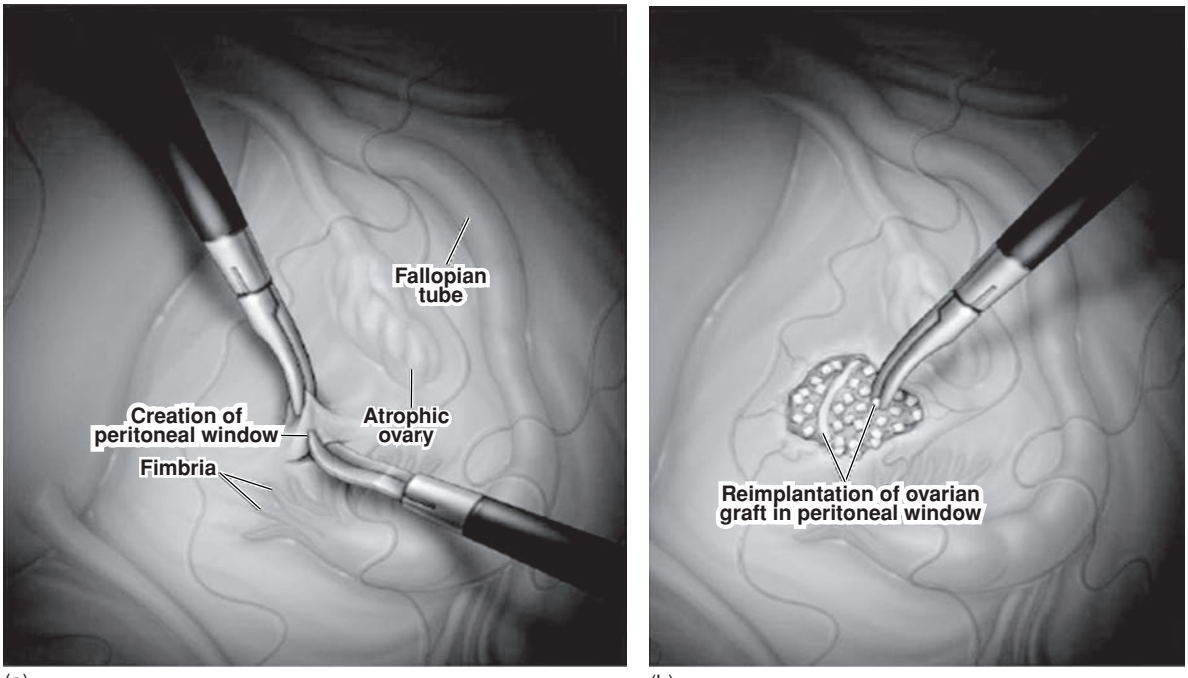



**Figure 29.2** (a) Creation of a peritoneal window close to the atrophic ovary. (b) Transplantation of ovarian cortex pieces into the peritoneal window. See plate section for color version.

### Re-implantation (Figure 29.2)

In two cases, we performed a first laparoscopy 7 days before re-implantation to create a peritoneal window by means of a large incision just beneath the right ovarian hilus, followed by coagulation of the edges of the window [6, 16]. The goal was to induce angiogenesis and neovascularization in this area.

A second laparoscopy was performed 7 days after creation of the peritoneal window. A biopsy of 4–5 mm in size was taken from each of the atrophic ovaries to check for the presence or absence of primordial follicles. The cryogenic vials were thawed at room temperature (between 21 and 23°C) for 2 min and immersed in a water bath at 37°C for another 2 min. Ovarian tissue was immediately transferred from the vials to tissue culture dishes (Becton Dickinson, NY, USA) in Leibovitz L-15 medium and subsequently washed three times with fresh medium to remove cryoprotectant before further processing. Thawed ovarian cortical tissue was placed in sterile medium and immediately transferred to the operating theater.

In the first case mentioned above, we pushed the large strip and 35 small cubes of frozen–thawed ovarian tissue into the furrow created by the peritoneal window, very close to the ovarian vessels and fimbria on the right side [16]. No suture was used. An extensive neovascular network was clearly visible in this space (Figure 29.3).

After a long discussion with the oncologists and patient, a third laparoscopy was proposed. At least

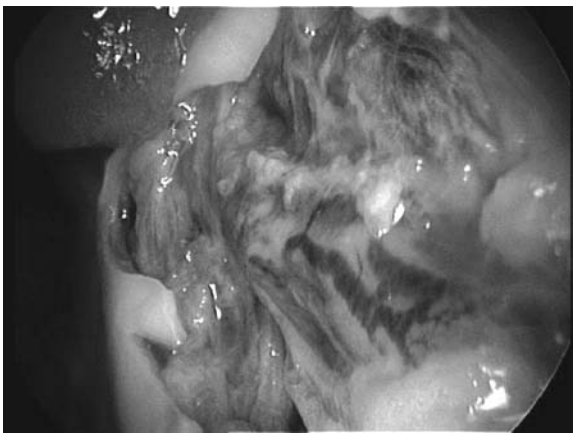


**Figure 29.3** An important vascular network is observed 7 days after the creation of the peritoneal window. See plate section for color version.

three reasons were given to justify the procedure: (1) to check the viability of the orthotopic grafts 4 months after transplantation by laparoscopic visualization and histological analysis; (2) to check for the absence of any cellular growth anomalies (peritoneal fluid, histology), the cortical strip and cubes having been biopsied before chemotherapy; and (3) to re-implant the remaining ovarian cortical cubes, by request of the patient, who was now aged 32 years. Indeed, if pregnancy had not ensued from the re-implanted tissue, she would have considered oocyte donation. A validated technique will probably not require so many surgical procedures in the future.

In the second case, a first laparoscopy was performed 7 days before re-implantation, not only to create a peritoneal window just beneath the left ovarian hilus, as previously described, but also to perform an ovarian incision along the longitudinal ovarian axis [6]. The edges of the window and the ovarian incision were coagulated in order to induce neovascularization in this area. Knowing from experimental data that the ovary itself, even if atrophic, may be an ideal site for re-implantation, we decided to simultaneously prepare two sites for re-implantation [5, 10, 28; M.-M. Dolmans *et al.*, personal communication]. Both sides were found to be effective as follicular development was demonstrated by vaginal ultrasound [6].

In the other cases, pieces of cryopreserved ovarian tissue were sutured to the medulla of the remaining ovary, either by laparotomy (Figure 29.4) or by laparoscopy (Figure 29.5).

Figure 29.4 shows transplantation of large ovarian cortical strips to the remaining ovary. Figure 29.5 illustrates the transplantation by laparoscopy.

By either laparotomy or by laparoscopy, the different steps are identical.

1. Decortication of the remaining ovary. The ovarian medulla is then denuded (Figure 29.4a).
2. If the ovarian strips are large enough, they are sutured on the ovarian medulla (Figure 29.4b)
3. If only small cubes or strips are available, they are placed on the ovarian medulla (Figure 29.5a) and covered by Interceed® (Johnson and Johnson, USA) (Figure 29.5b).

## Results from the literature

In 2000, Oktay and Karlikaya reported a laparoscopic transplantation of frozen–thawed ovarian tissue in a 29-year-old patient, who had undergone bilateral oophorectomy for a non-malignant disease [11]. Follicular development was demonstrated only once by ultrasonography 15 weeks after transplantation. The reason why this patient had undergone oophorectomy and re-implantation is not clear and no further data were available.

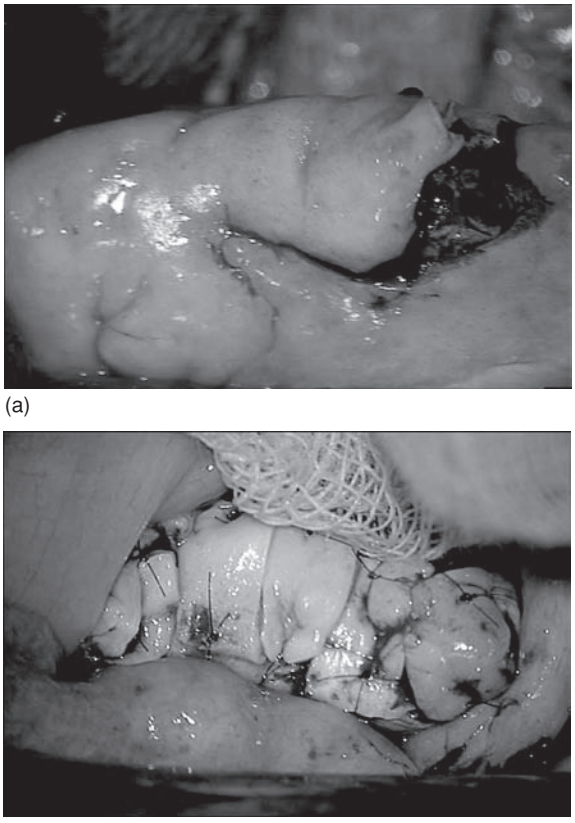
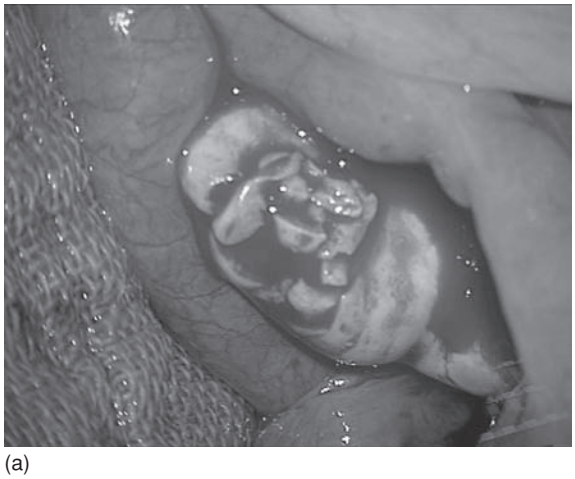
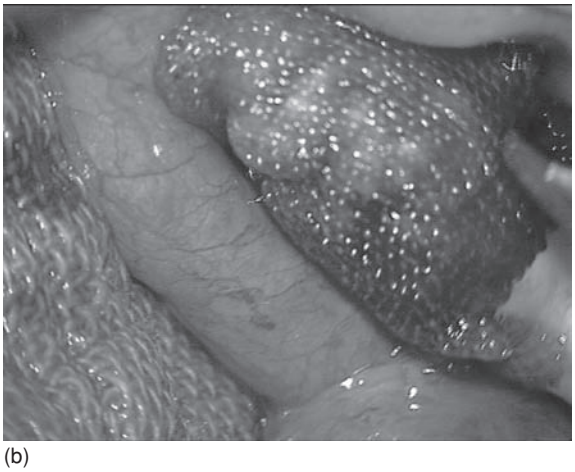


**Figure 29.4** (a) Decortication of the ovarian cortex from the remaining ovary. (b) Suture of the cryopreserved thawed cortical strips on the ovarian medulla. See plate section for color version.

Radford *et al.* reported a patient with a history of Hodgkin's disease treated by chemotherapy [13]. Ovarian tissue had been cryopreserved before receiving high doses of chemotherapy for a third recurrence of disease. Histological section of the ovarian cortical tissue revealed only a few primordial follicles due to the previous chemotherapy. Eight months after re-implantation, estradiol was detected and the follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels decreased. One month later, LH and FSH concentrations returned definitively to menopausal levels.



(a)



(b)

**Figure 29.5** (a) After decortication, the cryopreserved–thawed cortical cubes are placed on the ovarian medulla. (b) The ovarian cubes are covered with Interceed®. See plate section for color version.

We reported the first successful transplantation of cryopreserved ovarian tissue resulting in a pregnancy and live birth [16]. In 1997, a 25-year-old woman presented with clinical stage IV Hodgkin's lymphoma. According to Schilsky *et al*., the risk of POF after such a regimen in a woman of 26 years of age is more than 90% [29], while according to Wallace *et al*. and Lobo *et al*., the risk of subfertility after Hodgkin's treatment with alkylating agents is more than 80% [26, 30]. Ovarian tissue cryopreservation was undertaken before chemotherapy. After laparoscopy, the patient received hybrid chemotherapy from August 1997 to February 1998, followed by supradiaphragmatic radiotherapy (38 Gy).

In 2003, once the patient had been declared completely disease-free, re-implantation was carried out in orthotopic sites (see Donnez *et al*. for techniques [6, 16]). From 5 to 9 months after re-implantation, concentrations of FSH, 17 β-estradiol and progesterone showed the occurrence of ovulatory cycles. At 11 months, the patient became pregnant and subsequently delivered a healthy baby. This birth was announced in the *Lancet* in September 2004 [16].

In 2005, Meirow *et al*. also published details of a live birth after orthotopic autotransplantation of cryopreserved ovarian tissue in a patient with POF after chemotherapy [17]. Eight months after orthotopic transplantation, the patient spontaneously menstruated. Nine months after transplantation, she experienced a second spontaneous menstrual period. After a modified natural cycle, a single mature oocyte was retrieved and fertilized. Two days later, a four-cell embryo was transferred. The patient became pregnant from this embryo transfer and delivered a healthy infant weighing 3000 g.

Demeestere *et al*. reported a pregnancy after natural conception in a woman who had undergone orthotopic and heterotopic transplantation of cryopreserved ovarian tissue [19]. Unfortunately, this pregnancy, obtained by natural conception, ended in miscarriage at 7 weeks due to aneuploidy. The same team performed a second re-implantation to an orthotopic site in the same patient after cessation of graft secretion was evidenced [20]. The patient became pregnant and delivered a healthy baby. She recently delivered a second healthy child.

Silber *et al*. reported a pregnancy following re-implantation of cryopreserved ovarian tissue between monozygotic twins [21]. It should be noted, however, that the same woman had already delivered a first healthy baby after re-implantation of fresh tissue [21].

Andersen *et al*. described a series of six orthotopic re-implantations of cryopreserved ovarian cortex. In this series, two women became pregnant and delivered healthy infants [22]. Single mature oocytes were retrieved during optimized cycles, fertilized and transferred on day 3. One of the two women was later naturally pregnant and delivered a second healthy child.

In 2009, Piver *et al*. reported the birth of a healthy baby after orthotopic re-implantation of cryopreserved tissue [31]. The patient became pregnant naturally. Very recently, Sánchez-Serrano reported a twin pregnancy after an in vitro fertilization (IVF)

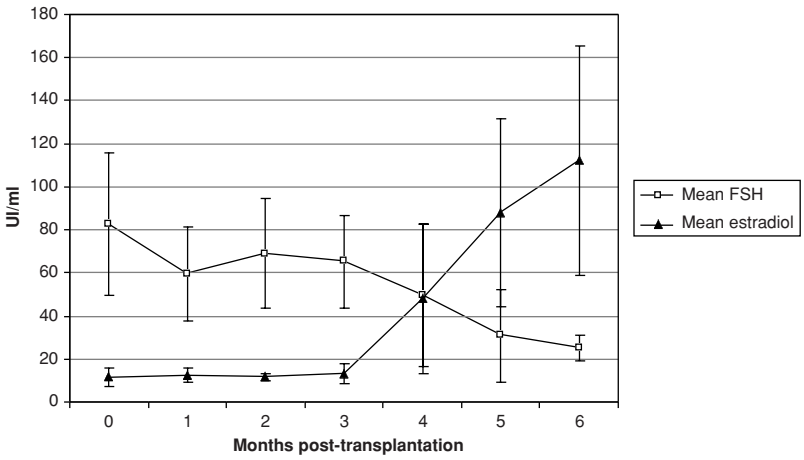



**Figure 29.6** Significant follicle stimulating hormone (FSH) decrease and estradiol increase are observed 4 months after ovarian tissue re-implantation. With permission from RMB Online.

procedure in a woman who have undergone orthotopic re-implantation of cryopreserved ovarian tissue [32].

## Restoration of ovarian function

In our series, orthotopic transplantation of ovarian tissue induced restoration of ovarian function [10]. Analysis of these cases raises some important points for discussion. First of all, in all cases, it took between $3^1/_2$ and 5 months after re-implantation before a follicle could be seen by ultrasonography and a decrease in FSH level observed (Figure 29.6). The process of folliculogenesis takes around ~4–6 months, during which time the oocyte and surrounding somatic cells undergo a series of changes that eventually result in the development of a large antral follicle, capable of producing a mature oocyte [33]. Thus, the appearance of the first follicle originating from grafted tissue 4–5 months after re-implantation, proved by laparoscopy in one case, is totally consistent with the expected time course. This time interval between implantation of cortical tissue and the first estradiol peak is also consistent with data obtained from sheep [34, 35] and human beings, although some variations may be observed. Indeed, the delay between transplantation and follicular development was found to vary from 6 weeks to 8 months. Such a variation could be explained by a difference in follicular reserve at the time of cryopreservation.

Another very interesting finding in our series was the persistence of relatively high FSH levels during the follicular phase. The FSH levels remained as high as 25 mIU/ml during the follicular phase until ovulation, and then decreased to <15 mIU/ml during the luteal phase. This may constitute an argument against the use of gonadotropin injections. The relatively high FSH levels may be explained by the relatively low number of surviving primordial follicles in the graft. The patient should be considered a poor responder, with reduced inhibin B secretion. These results are in agreement with those obtained in sheep by Campbell *et al.* [36].

A further significant observation was the return to an FSH level of >25 mIU/ml immediately after each menstrual bleed, which supports the theory suggested by Baird *et al.* that some inhibitory mechanisms, such as inhibin B or anti-Müllerian hormones (AMH) normally produced by developing follicles in intact human ovaries, are probably almost non-existent in transplanted tissue [35]. After transplantation, the patient would have been regarded a poor responder because, of the 500–1000 primordial follicles that would have been transplanted, more than 50% would have been lost owing to hypoxia [16].

## The crucial issue of revascularization

Van Eyck *et al.* recently characterized the oxygen environment in human ovarian xenografts in the early post-grafting period (up to day 21) using electron paramagnetic resonance oximetry [37]. This technique allows sensitive, non-invasive and repeated measurement of $PO_2$ in vivo. Before day 5, grafts were exposed to hypoxia. From day 5 to day 10, progressive reoxygenation was observed, suggesting an active process of graft revascularization.

Using a combined method of perfusion study and double immunohistochemical staining of human

and murine vessels, the same team evaluated the revascularization process of human ovarian tissue in this model [38].

On day 5, reperfusion of ovarian grafts was initiated by host angiogenesis, as evidenced by the appearance of murine neovessels penetrating from the periphery and colocalized with perfused areas. By day 10, the center of the fragments was perfused and ovarian graft angiogenesis contributed to the vascular pattern of the ovarian transplants.

Host and graft angiogenesis thus both appear to contribute to post-transplantation vascular behavior and could be potential targets to improve the mechanisms leading to perfusion of grafts with the aim of reducing the avascular period.

## Is there still a place for heterotopic transplantation?

There are only a few existing reports on this subject. Callejo *et al.* evaluated the long-term function of cryopreserved heterotopic grafts, but no conclusions could be drawn since the patient was perimenopausal at the time of ovarian biopsy for cryopreservation [23].

In 2004, Kim *et al.* reported a case of a 37-year-old woman who underwent heterotopic (rectus and pectoralis muscle) transplantation of cryopreserved ovarian tissue [14]. By 14 weeks of transplantation, restoration of endocrine function was demonstrated but, approximately 28 weeks after transplantation, cessation of ovarian function was evidenced by very high FSH levels (62–99 mIU/L) and very low estradiol levels.

The same year, Oktay *et al.* reported transplantation of frozen–thawed ovarian tissue beneath the skin of the abdomen [12]. A 4-cell embryo was obtained from 20 oocytes retrieved from an ovarian graft, but no pregnancy occurred after transfer. Oocyte quality might have been compromised by transplantation to a heterotopic site.

Kim *et al.* reported in 2004 on the heterotopic transplantation of cryopreserved ovarian tissue in a patient cured of squamous cell carcinoma of cervix [14]. Tissue was transplanted to two heterotopic sites: abdominal (rectus muscle) and breast site (pectoralis muscle). Growing follicles were seen in the abdominal site from 14 weeks after transplantation, but ovarian function ceased around 28 weeks after transplantation.

Wolner-Hanssen *et al.* reported on the subcutaneous transplantation of frozen–thawed tissue to the forearm in 2005 [9]. Two follicles developed, but only to a maximum diameter of 12.6 and 6.7 mm, respectively, and the tissue survived 7 months.

Recently, Kim *et al.* reported on the heterotopic autotransplantation of cryopreserved ovarian tissue in four patients (three with cervical cancer and one with breast cancer) [15]. Thawed ovarian fragments were transplanted into a space between the rectus muscle and the rectus sheath. Recovery of ovarian function was evidenced in 3 patients by hormone profiles obtained between 12 and 20 weeks after transplantation, but this only lasted 3–5 months. These three patients subsequently underwent a second transplantation. Long-term ovarian function (15–36 months) was then established. Ovarian grafts were stimulated daily with FSH, until a dominant follicle size of 14–16 mm was reached. During a 27-month follow-up period in 2 patients, 6 oocytes were retrieved (1 germinal vesicle [GV], 4 metaphase-I [MI], 1 metaphase-II [MII]). The MI oocytes were subjected to in vitro maturation. All 4 MII oocytes then fertilized and developed to cleavage-stage embryos (up to 6 cells on day 3) before being frozen for transfer to a surrogate.

Papers describing heterotopic transplantation have all reported follicular development, but with follicles always $< 16$ mm in size [14, 19]. As stressed by Wolner-Hanssen *et al.* and Oktay *et al.*, differences in temperature and pressure could interfere with follicular development in heterotopic sites [9, 12].

In our opinion, there is no place for heterotopic transplantation if the goal is to restore fertility.

## Conclusion

Approximately one third of young women exposed to chemotherapy develop ovarian failure. In 2010, we believe it is our ethical responsibility to propose cryopreservation of ovarian tissue to all adolescents and young women under Institutional Review Board (IRB) protocols having to undergo chemotherapy with alkylating agents.

In conclusion, the 11 live births obtained after transplantation of frozen–thawed ovarian tissue in humans give hope to young cancer patients, but there is still much work to be done. Research programs need to determine whether active angiogenesis can be induced to accelerate the process of neovascularization in grafted tissue, if isolated human follicles can be grafted or, indeed, if microvascular re-anatomosis of an entire cryopreserved ovary is a valuable option.

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38. **Van Eyck AS**, **Bouzin C**, **Feron O** *et al.* Both host and graft vessels contribute to revascularization of xenografted human ovarian tissue in a murine model. *Fertil Steril* 2010; **93**(5): 1676–85.

**Figure 1.1** John Hunter (1728–93). The first reported person to successfully perform artificial insemination in a human.

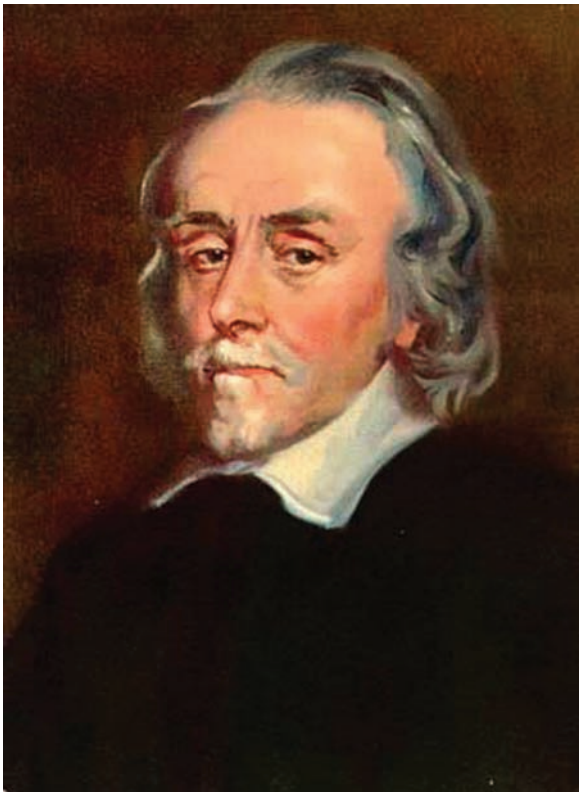


**Figure 1.2** William Harvey (1578–1657). The first person to describe the egg as responsible for the production of all creatures.

**Figure 1.3** Anton van Leeuwenhoek (1632–1723). The first person to study animal and human sperm under microscopes, which he constructed himself.

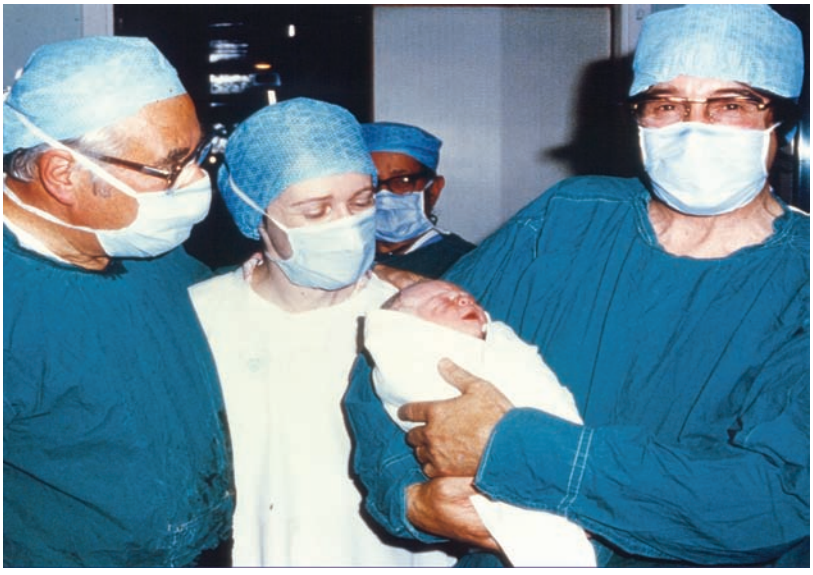


**Figure 1.6** Patrick Steptoe and Robert Edwards at the birth of the world's first in-vitro fertilization conceived baby – Louise Brown – on July 25, 1978. Courtesy of Bourn Hall Clinic.

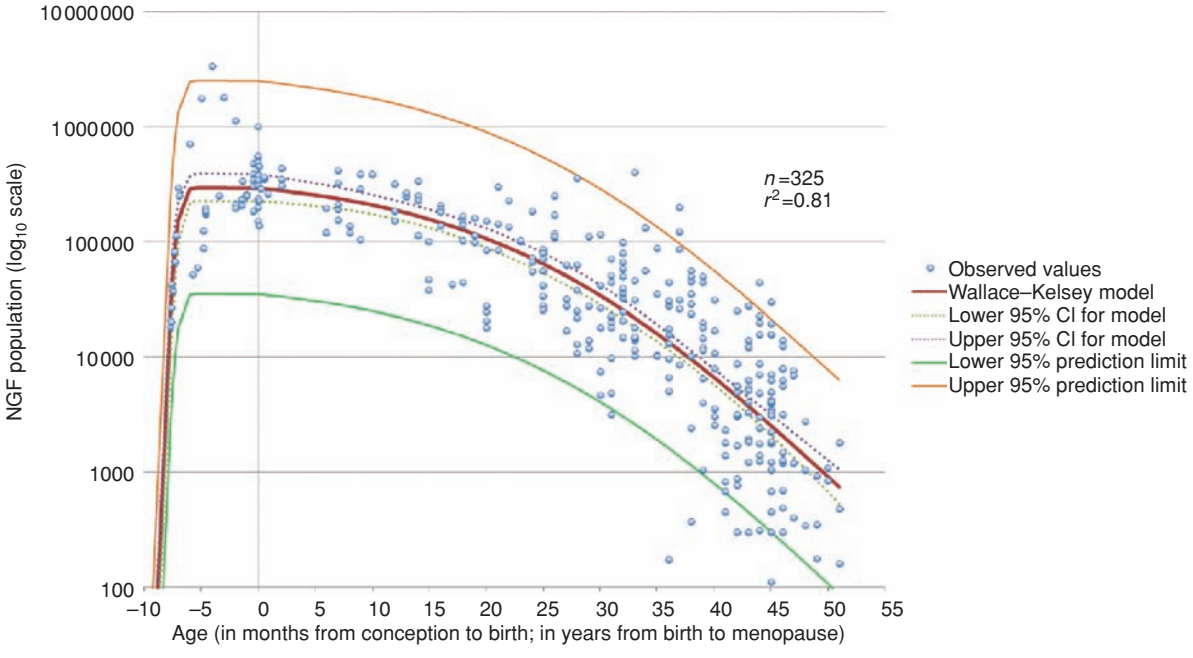



**Figure 2.1** The best model for the establishment of the non-growing follicle (NGF) population after conception and the subsequent decline until age at menopause is described by an ADC model. The model has a correlation coefficient $r^2 = 0.81$, fit standard error = 0.46 and F-value = 364. The figure shows the dataset ($n = 325$), the model, the 95% prediction limits of the model and the 95% confidence interval (CI) for the model. The horizontal axis denotes age in months up to birth at age 0 and age in years from birth to 51 years. From Wallace and Kelsey [8].

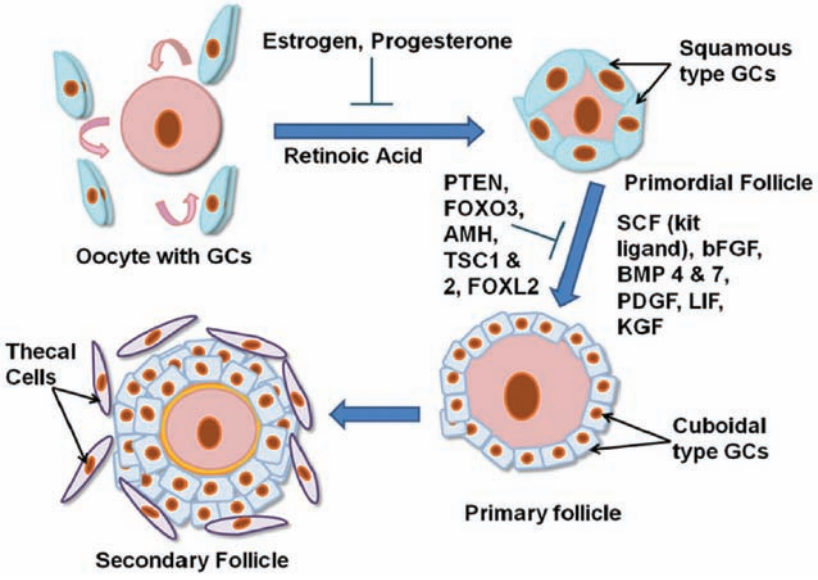



**Figure 10.1** Primordial follicle to secondary follicle formation (gonadotrophin independent phase): The oocyte, under the stimulatory effects of retinoic acid, recruits squamous type granulosa cells (GCs) to form the primordial follicle. Steroid hormones inhibit the formation of primordial follicles. Under the activating effects of stem cell factor (SCF)/kit ligand, basic fibroblast growth factor (bFGF), bone morphogenetic proteins 4 and 7 (BMP-4 and -7), platelet derived growth factor (PDGF), leukemia inhibitory factor (LIF) and keratinocyte growth factor, the primordial follicle further develops into the primary follicle with accompanying morphological changes of the supportive cells, i.e. from a monolayer of squamous type GCs to cuboidal type GCs. Thecal cells then surround the now multiple layers of GCs which encloses the developing oocyte to form the secondary follicle. Repressive signals like phosphatase and tensin homolog (PTEN), Foxo3 and anti-Müllerian hormone (AMH), tuberous sclerosis complexes (TSC) 1 and 2 and FOXL2 inhibits the development of primordial follicles to secondary follicles.

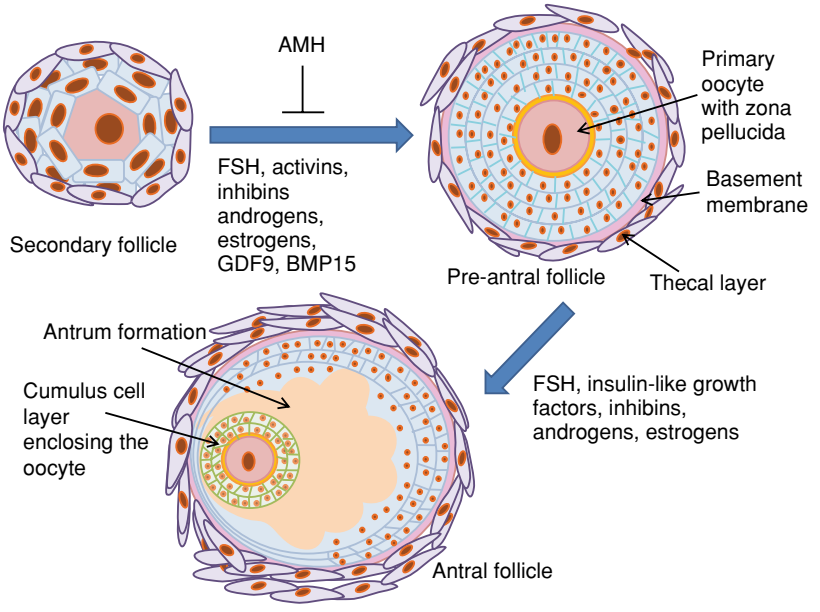



**Figure 10.2** Pre-antral phase to antral phase transition. As the secondary follicle develops, it becomes more gonadotrophin sensitive with the expression of gonadotrophin receptors. Under the activation of follicle stimulating hormone (FSH) and the effects of activins and inhibins, androgen synthesis with estrogen production take place, resulting in the development of the secondary follicle into the pre-antral follicle. Anti-Müllerian hormone (AMH) has been shown to suppress early stages of follicular growth and onset of responsiveness to FSH in vitro, exerting a controlling influence on the rate at which follicles become available for pre-ovulatory development. Enhancing effects from growth differentiation factor (GDF-9), bone morphogenetic protein 15 (BMP-15) and insulin-like growth factors enables the pre-antral follicle to develop further into the antral follicle with the formation of the antrum. At the same time, the GCs proliferate and differentiate into the cumulus cell layer which encloses the developing oocyte. With the antral cavity filled with follicular fluid, the pre-antral follicle now becomes the antral follicle.

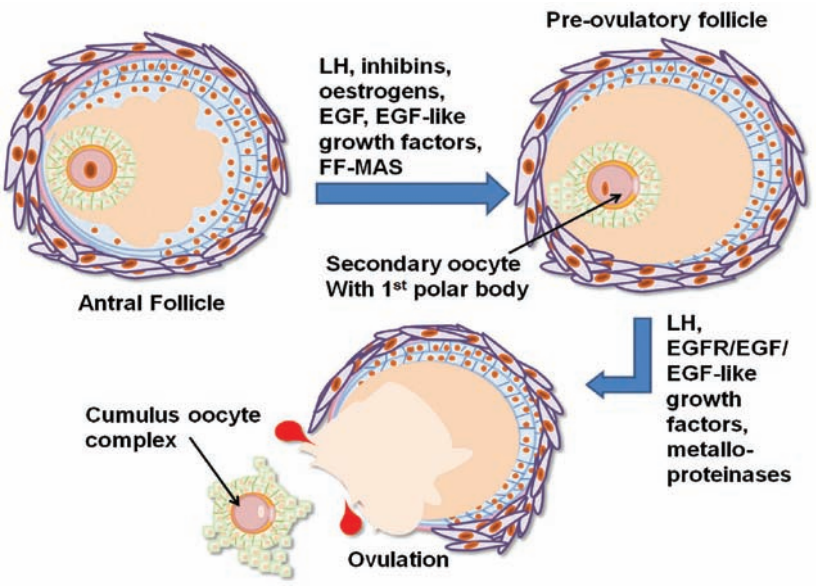



**Figure 10.3** Antral phase to ovulation: The antral follicle progresses to develop into the pre-ovulatory follicle under the stimulation of luteinizing hormone (LH). This transition is augmented by epidermal growth factors (EGF), EGF-like growth factors and follicular fluid meiosis-activating sterol (FF-MAS). A critical step in this transition is the completion of meiosis I in the maturing oocyte with the extrusion of the first polar body. This ensures that the oocyte achieves meiotic competence prior to ovulation. Under the LH surge and increased expression of metalloproteinases and proteolytic enzymes, the pre-ovulatory follicle subsequently ruptures and ovulation takes place with the release of the cumulus oocyte complex. The follicle then collapses and proceeds to form the corpus luteum.

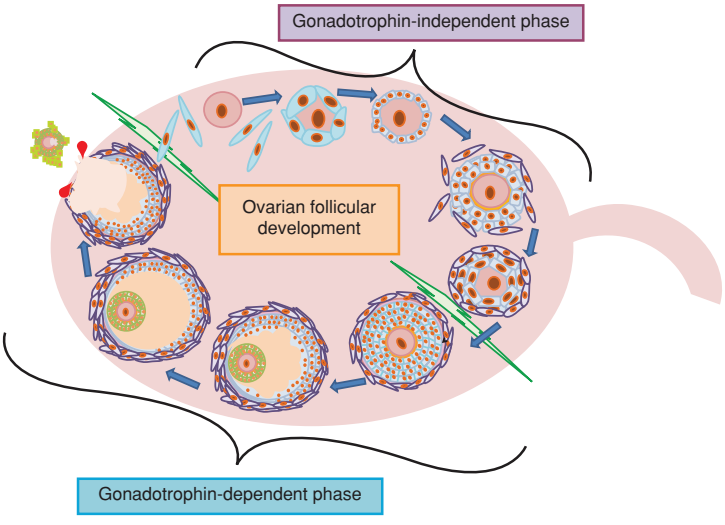



**Figure 10.4** In the ovary, follicular development constitutes of a gonadotrophin-independent and a gonadotrophin-dependent phase. Coordination of multiple growth factors, hormones and biochemical molecules signaling at timely intervals with the activation of several pathways, e.g. PI3K, JAK/STAT, WNT/β-catenin and MAPK between the growing oocyte, surrounding somatic cells and wider endocrine system ensures optimum follicular development with the release of a meiotically and developmentally competent oocyte.

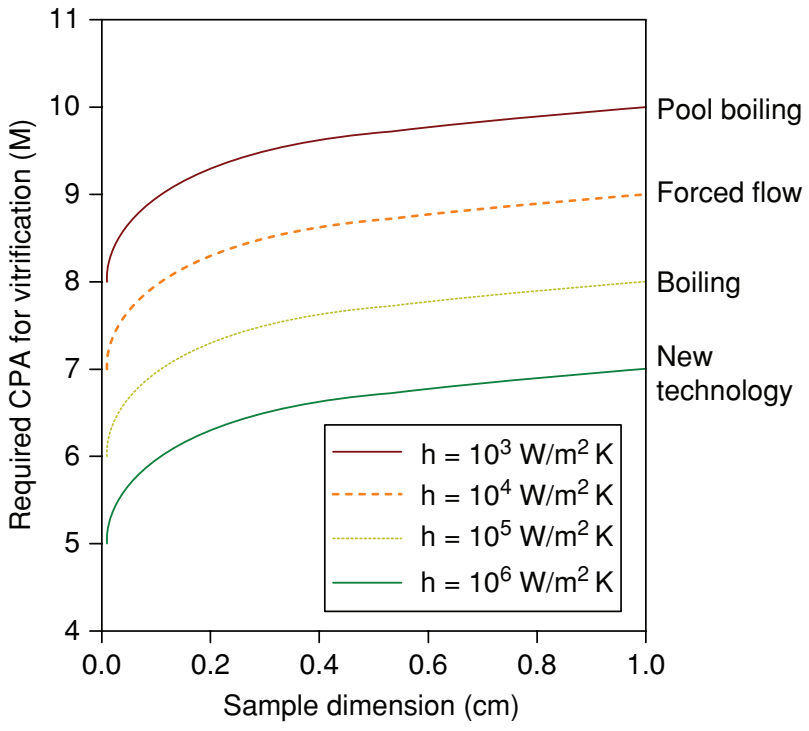



**Figure 11.1** The effect of sample size on the cryoprotectant agent (CPA) concentration required to achieve vitrification at various cooling rates. Plunging samples into liquid nitrogen ($LN_2$) is an example of a pool boiling approach. Flowing $LN_2$ over a sample is an example of a forced flow boiling approach. Oscillating heat pipe (OHP) technology (data from Jiao *et al.* [41]) is an example of new technologies that are being developed to increase our ability to apply vitrification approaches to biological samples.

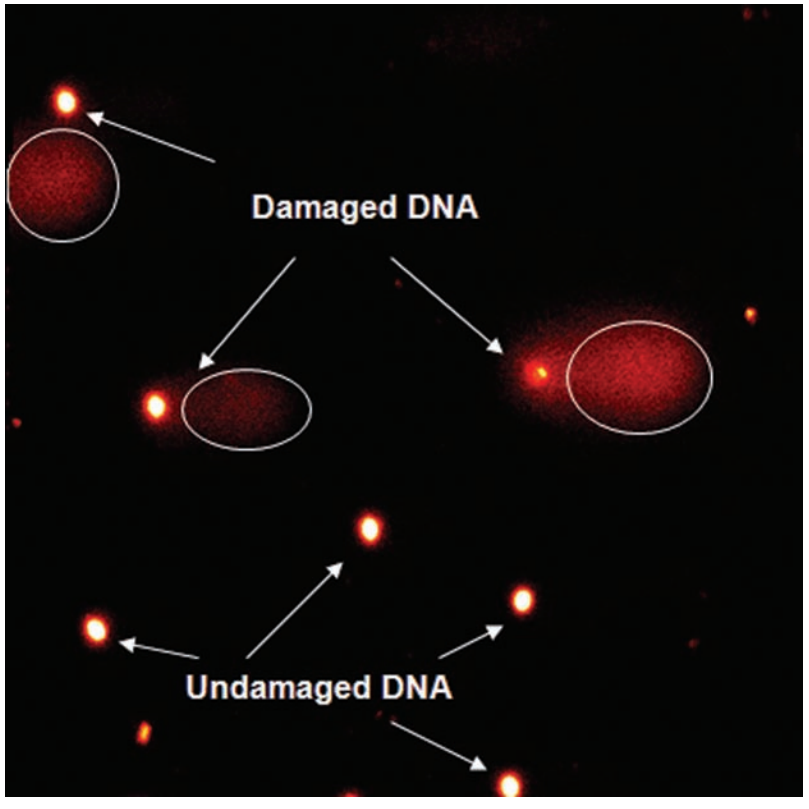



**Figure 14.1** Damaged and undamaged DNA after warming. Fluorescent staining was performed using SYBR green stain (working concentration 1:200). In healthy cells, the fluorescence was confined to the nucleoid: undamaged DNA is supercoiled and does not migrate very far from the nucleoid. In cells that have incurred damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the nucleoid and form the so-called "comet-tail" (circled).

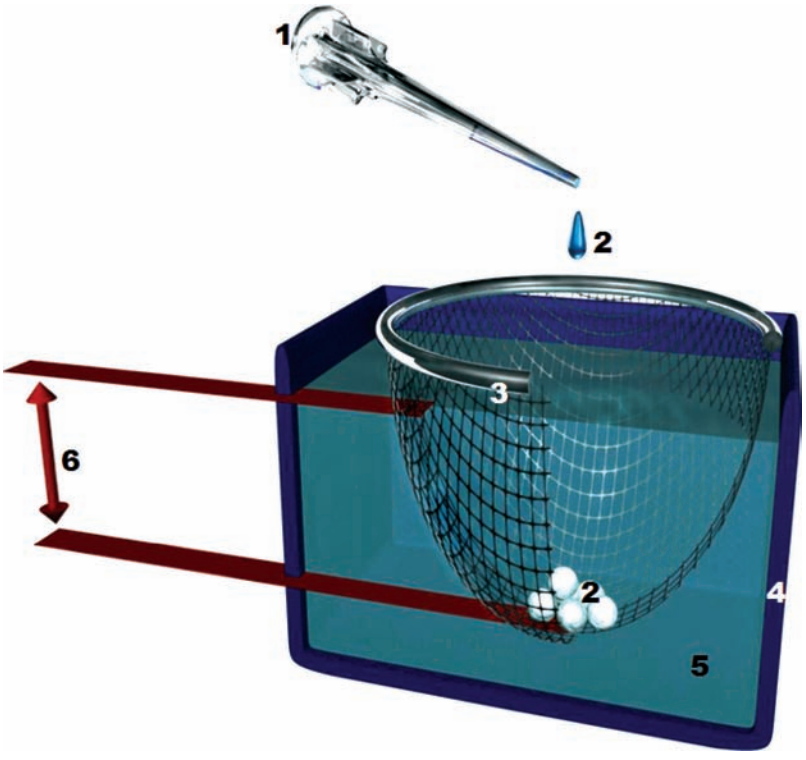



**Figure 14.3** Scheme of the spermatozoa vitrification procedure. (1) Single channel pipettes with adjustable volume (30 μl). (2) Spermatozoa suspension. (3) Strainer. (4) Foam box. (5) Liquid nitrogen. (6) Distance between bottom of strainer and surface of liquid nitrogen (minimum 3 cm). With permission from Isachenko *et al.* [115].

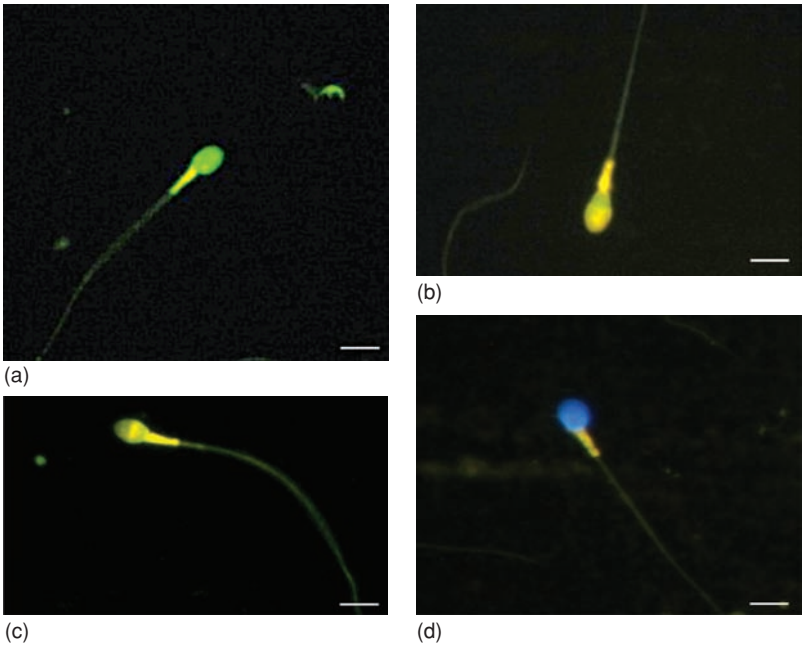



**Figure 14.4** Example of non-capacitated (a), capacitated (b), acrosome-reacted (c) and non-viable (d) spermatozoa. At least 200 spermatozoa were observed in each plate and 3 patterns were identified (see Figure 14.2). (a) A uniform fluorescence on the head of the spermatozoa (non-capacitated spermatozoa). (b) A band of fluorescence diminished in the post-acrosomal region and a relatively shining fluorescence in the acrosomal region (capacitated spermatozoa). (c) A fluorescence in the complete head of the spermatozoa, except a tenuous band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa). The slides were viewed using a Zeiss Axiolab Epifluorescence microscope that was equipped with an excitation/emission filter of 485 nm/520 nm under × 400 magnification. The non-viable spermatozoa were observed with the filter set 09 (450–490 nm). The dead spermatozoa displayed a pattern of blue fluorescence in the whole head (Figure 14.2d). With permission from Isachenko *et al.* [115].

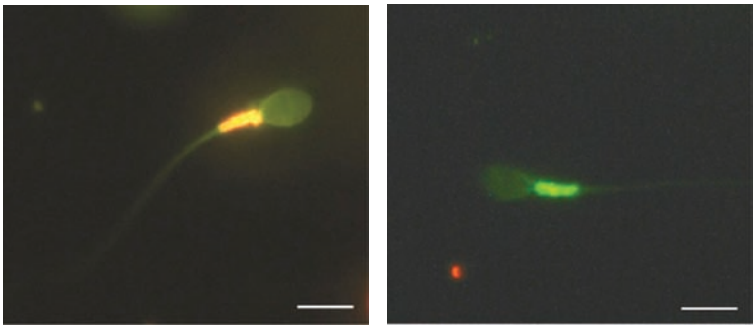



**Figure 14.7** Example of undamaged (a) and damaged mitochondria (b). In undamaged mitochondria the mitochondrial membrane potential (M $\Delta\Psi$) is intact and the JC-1 reagent aggregate inside of the non-damaged mitochondria and fluoresces red. In our case, the midpiece is yellow, as expected for the red fluorescence from JC-1 aggregates merging with the green fluorescence of JC-1 monomer dispersed throughout the cell plasma membrane. In damaged mitochondria, the M $\Delta\Psi$ is broken down and the JC-1 reagent disperses though the entire cells and fluoresces green. The changes in M $\Delta\Psi$ is measured using an unique fluorescent cationic dye, 5,5′,6,6′-tetachloro-1–1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1.

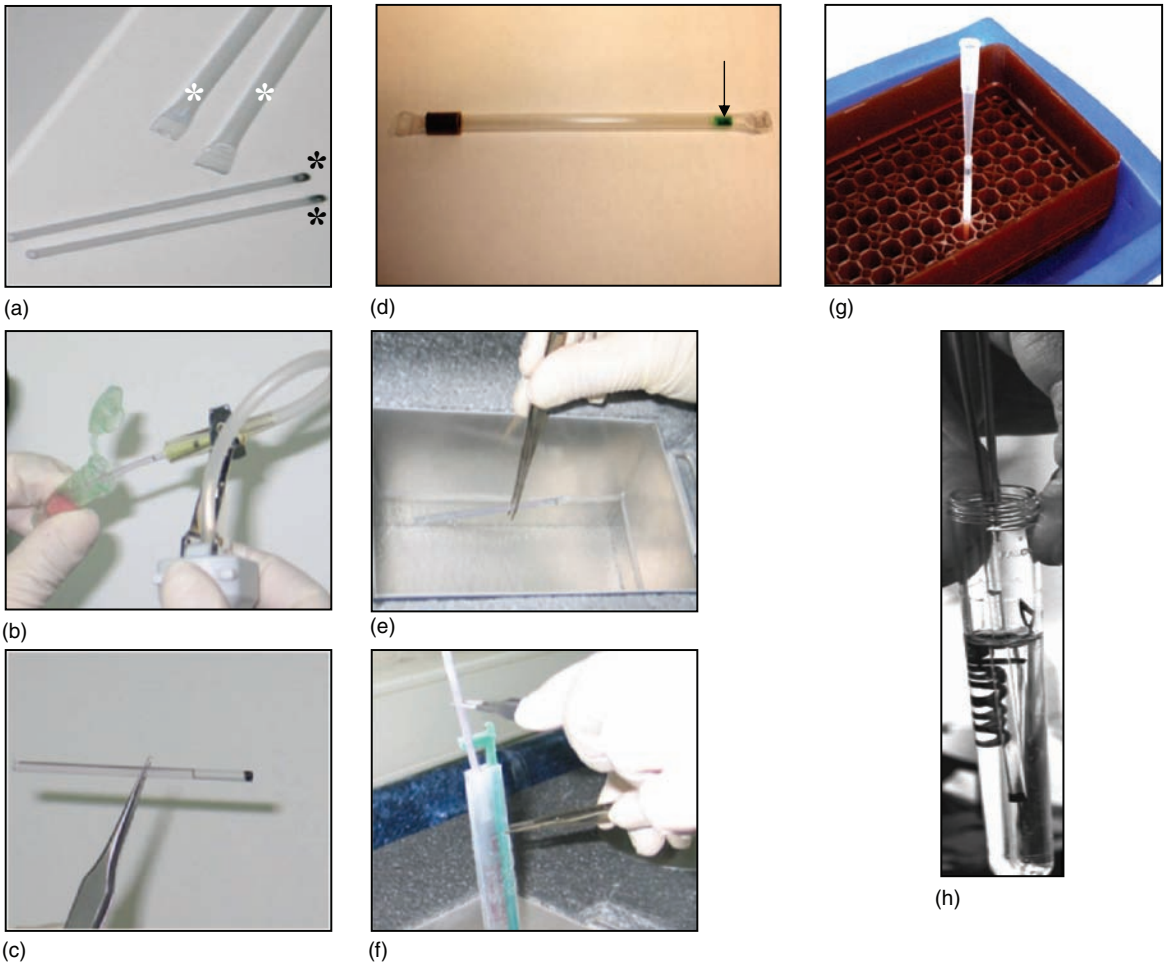



**Figure 14.12** The vitrification procedure for big volume of spermatozoa suspension. (a) The 0.25 ml plastic straws are halved and dark-marked from one side (dark asterisks) and a 0.5 ml packaging straw (white asterisks). (b) The non-marked side of the half-straw is filled with spermatozoa suspension. (c) A 0.25 ml half-straw, hermetically closed from both sides, is filled with spermatozoa suspension. (d) Holding it in a horizontal position, the 0.25 ml half-straw filled with spermatozoa suspension is marked from one side (arrowed) and placed into a 0.5 ml packaging straw, closed from both sides. (e) Using tweezers and keeping it in a horizontal position, this closed packaging system is directly immersed into liquid nitrogen ($LN_2$) and submerged for over 5 s to prevent the flow of spermatozoa suspension spreading into packaging straw. (f) The vitrified sample is stored in $LN_2$. Using tweezers, the dark-marked part of the Sealed Pulled Straw (SPS), approximately 1.0–1.5 cm, is removed from the $LN_2$ and the end of packaging straw is cut. (g) With the help of a 200 μl pipette tip (Eppendorf AG, Hamburg, Germany), the suspension-filled fixed straw is quickly removed from the packaged straw and (h) immersed into a 15 ml plastic tube containing 6 ml of human tubal fluid (HTF) and human serum albumin (HSA) prewarmed to 37°C with gentle agitation to accelerate the melting and removing of content.

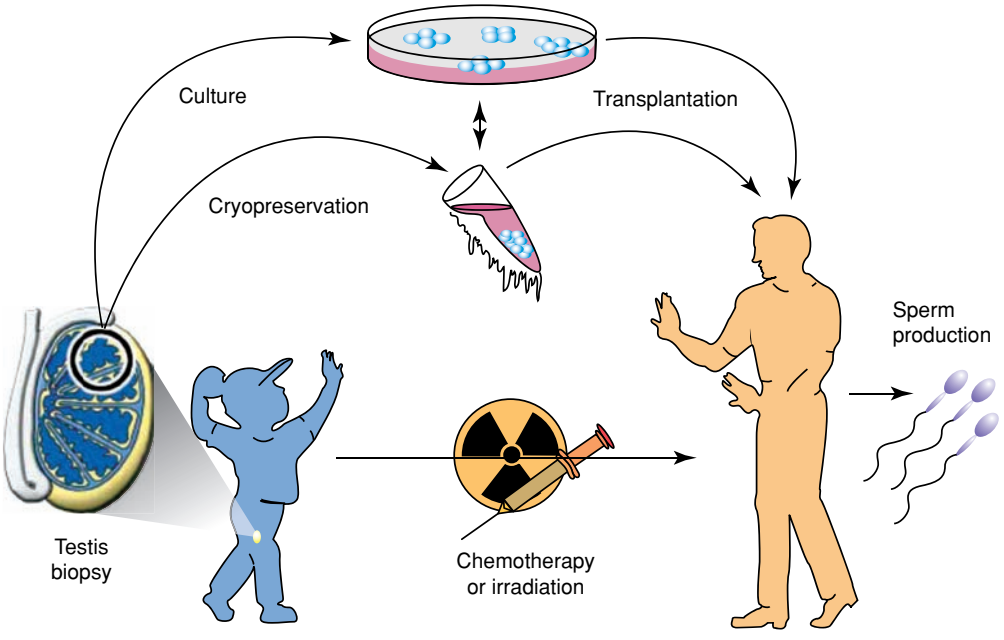



**Figure 15.1** Male germline stem cell preservation. Before treatment for cancer by chemotherapy or irradiation, a boy could undergo a testicular biopsy to recover stem cells. The stem cells could be cryopreserved or, after development of the necessary techniques, could be cultured. After treatment, the stem cells would be transplanted to the patient's testes for the production of spermatozoa. From Brinster [14] with permission.

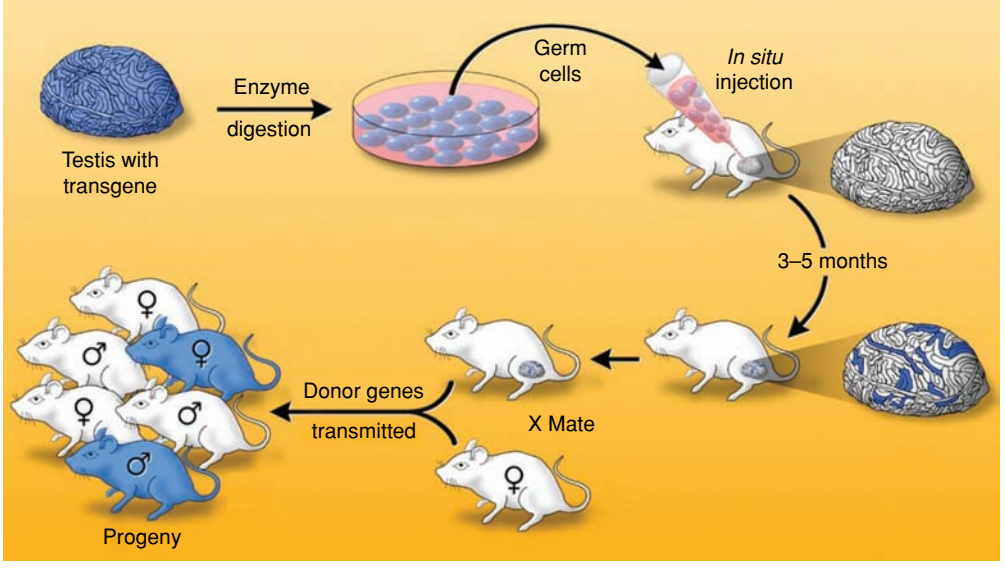



**Figure 15.2** Testis cell transplantation method. A single-cell suspension is produced from a fertile donor testis. The cells can be cultured or microinjected into the lumen of seminiferous tubules of an infertile mouse. Only a spermatogonial stem cell can generate a colony of spermatogenesis in the recipient testis. When testis cells carry a reporter transgene that allows the cells to be stained blue, colonies of donor cell-derived spermatogenesis are identified easily in the recipient testes as blue stretches of tubule. Mating the recipient male to a wild-typed female produces progeny, which carry donor genes. From Brinster [11] with permission.

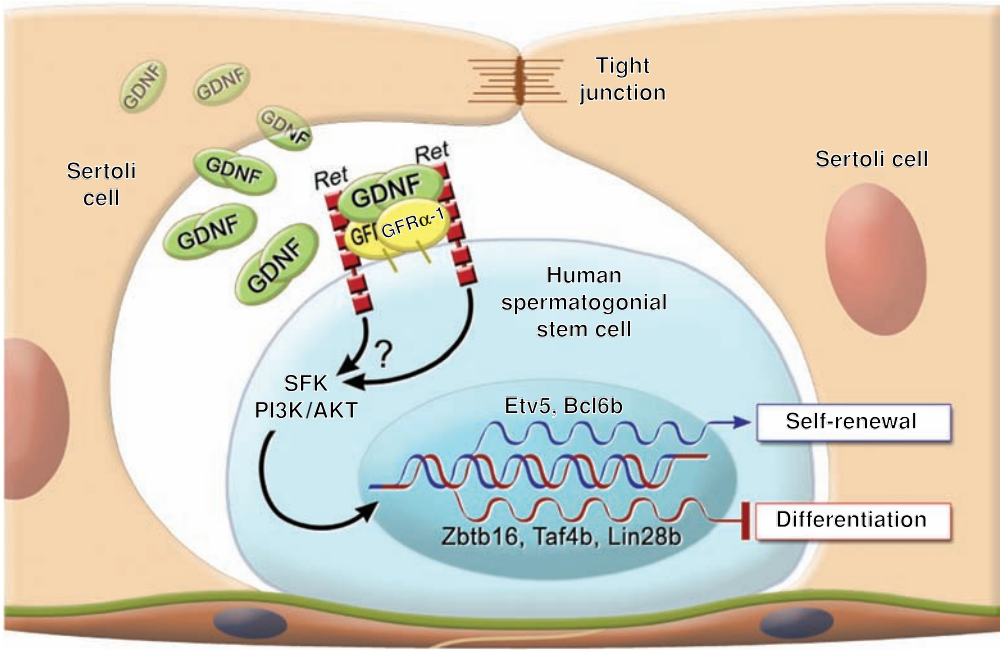
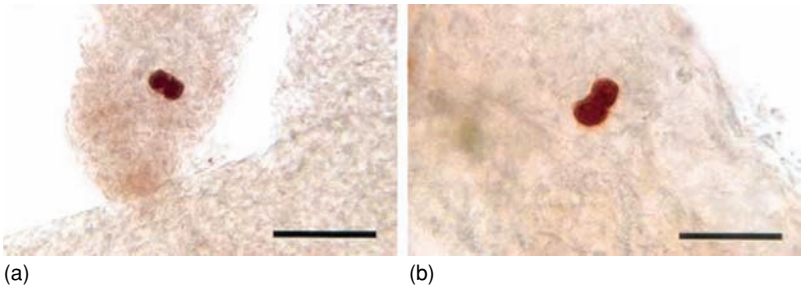



**Figure 15.3** A proposed model of human spermatogonial stem cell (SSC) self-renewal regulation by glial cell line-derived neurotrophic factor (GDNF), which has been demonstrated to have an essential role in regulating rodent SSC self-renewal. The model is similar to those suggested for mouse SSC self-renewal. In this model, GDNF binds to RET and the GFR$\alpha$1 coreceptor with possible intracellular protein kinase signaling through SFK and PI3K/AKT downstream pathways to regulate the expression of specific genes, such as Etv5 and Bcl6b, which are involved in SSC self-renewal. However, other genes not regulated by GDNF (e.g. Zbtb16, Taf4b and Lin28), are likely controlled by different signals and may block differentiation but not be involved directly in self-renewal. Genes for these regulatory molecules have been shown to be highly expressed in pre-pubertal human spermatogonia, mouse gonocytes and mouse SSCs. The basement membrane (green), on which the SSC rests, is generated by the peritubular myoid cells (dark brown) and Sertoli cells (tan). From Wu *et al.* [27] with permission.



(a) (b)

**Figure 15.4** Detection of human germ cells transplanted into seminiferous tubules of recipient mouse testes using a baboon testis specific antibody that identifies human spermatogonia. (a) Donor human spermatogonia in mouse tubule 4 months after transplantation of cryopreserved cells. This panel shows that cryopreserved cells also colonize mouse testes as observed with freshly transplanted cells. (b) Donor human spermatogonia in mouse testis 5 months after transplantation. These donor cells were transplanted without cryopreservation. Bar = 100 $\mu$m (a) and 40 $\mu$m (b). From Nagano *et al.* [17] with permission from Elsevier Science, Inc. © 2002 American Society for Reproductive Medicine.

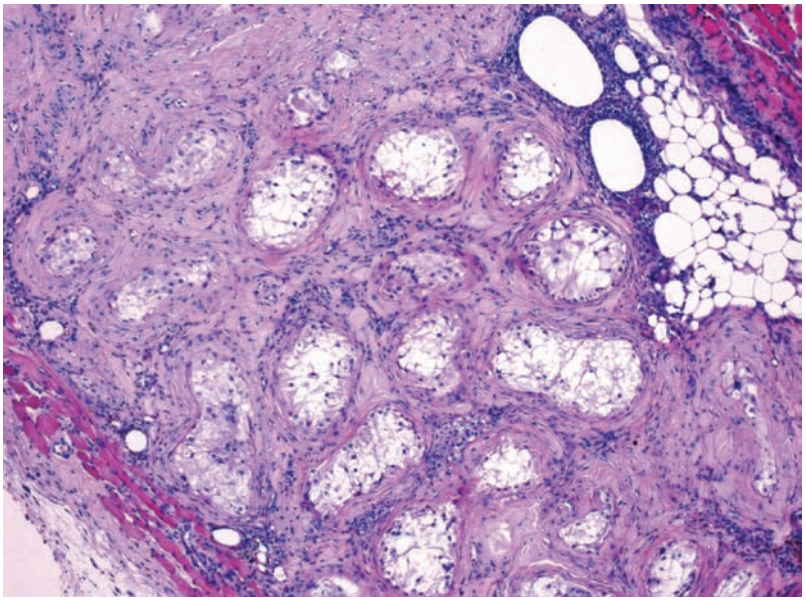


**Figure 16.1** Histological appearance (hematoxylin/eosin sections) of donor testicular tissue from a 44-year-old man after 3 weeks' orthotopic xenografting at ×200 magnification. Most tubules show degenerative changes, i.e. sclerosis, while the rest contain mainly Sertoli cells.

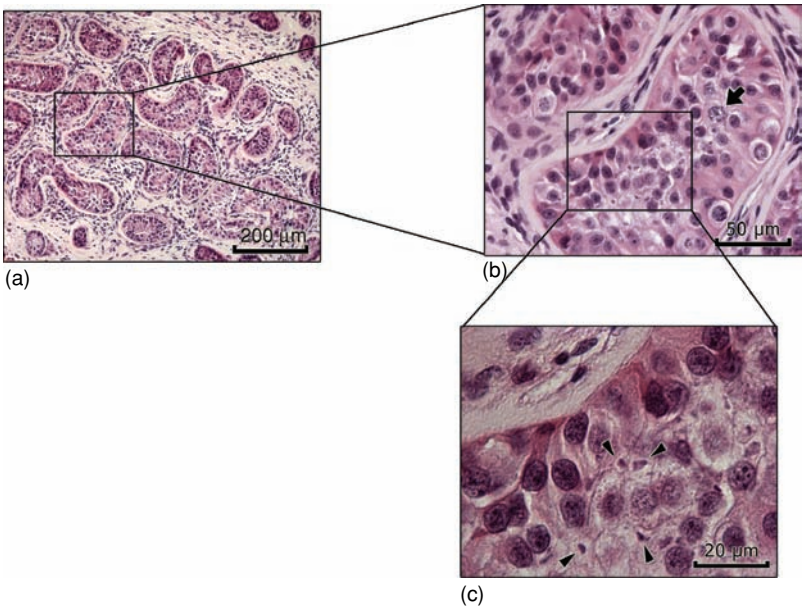



**Figure 16.2** Histological appearance (hematoxylin/eosin sections) of donor testicular tissue from a 12-year-old boy (a) after 6 months' orthotopic xenografting at ×200 magnification; (b) showing pachytene spermatocytes (arrow) and spermatid-like cells (inset) at ×400 magnification; and (c) spermatid-like cells at ×1000 magnification.

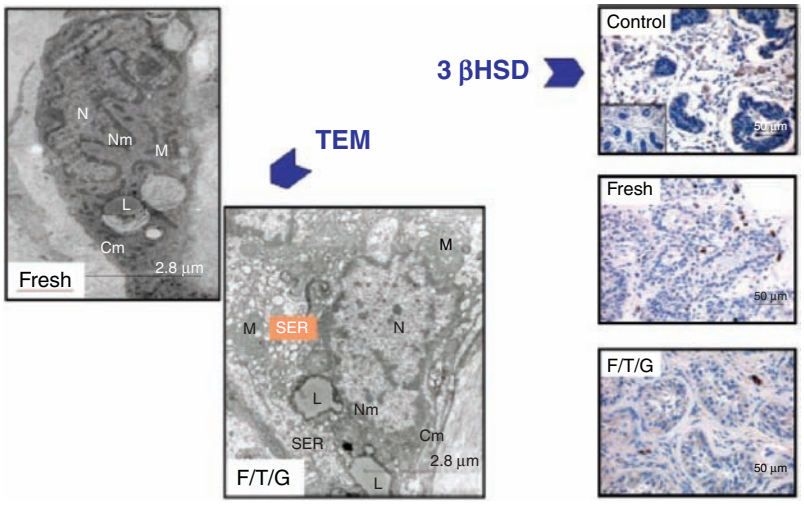



**Figure 16.3** Steroidogenic activity in Leydig cells evaluated by transmission electron microscopy (TEM) (left) and immunohistochemistry (IHC) (right). The TEM shows fresh and frozen/thawed/grafted Leydig cells showing intact structures of nuclear and cytoplasmic components and activity. Magnification ×12 000. Bm, basement membrane; Cm, cell membrane; F/T/G, frozen, thawed and grafted for 6 months; L, lipid droplets; M, mitochondria; N, nucleus; Nm, nuclear membrane; SER, smooth endoplasmic reticulum: site of conversion of pregnenolone to testosterone. The IHC shows fresh and frozen/thawed/grafted Leydig cells that are stained for 3 β-hydroxysteroid dehydrogenase (3 βHSD), converting pregnenolone to progesterone.

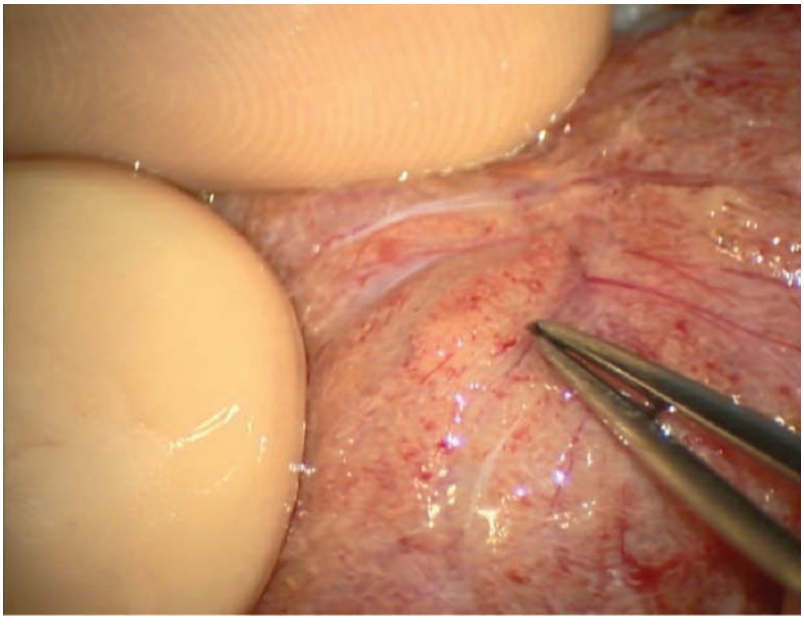


**Figure 17.2** Intraoperative picture of tubules more likely to harbor spermatogenesis, as indicated by forceps.

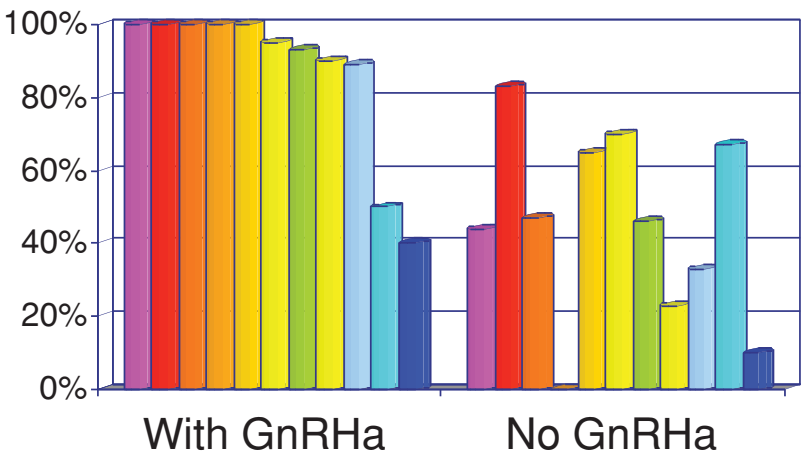



**Figure 18.2** Percentage of women with a return of ovarian function following chemotherapy. Each study is a different color. GnRHa, gonadotropin-releasing hormone agonist.

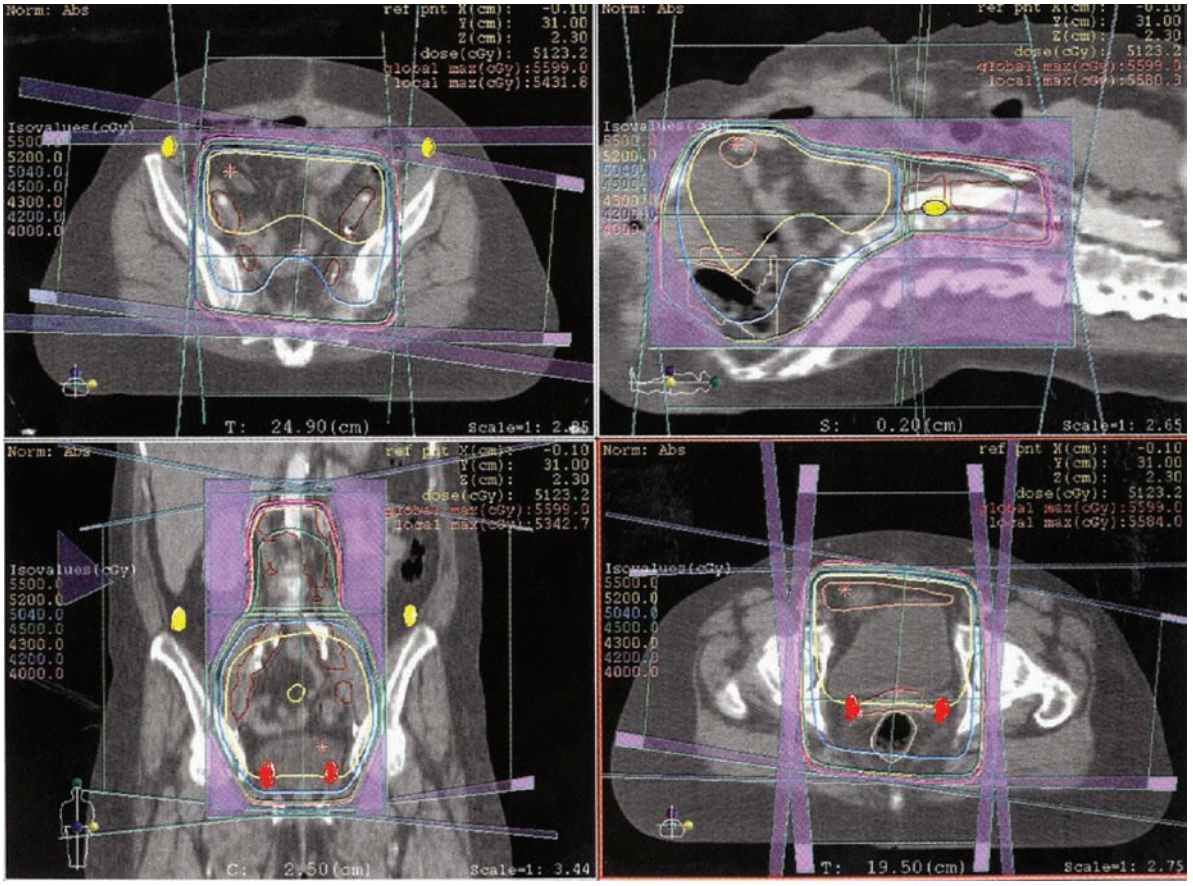



**Figure 19.1** Radiation field.

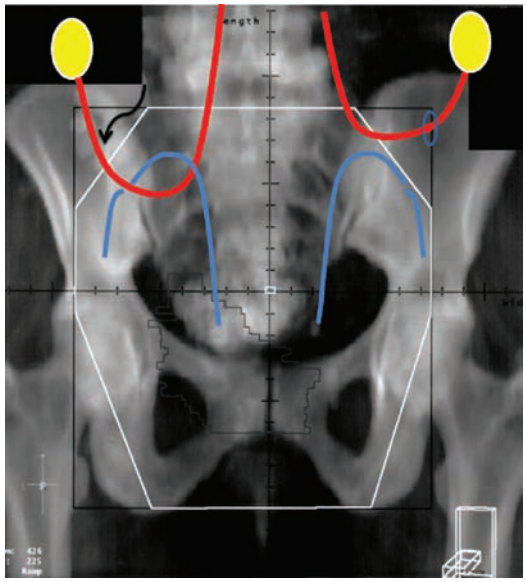


**Figure 19.2** Surgical anatomy.

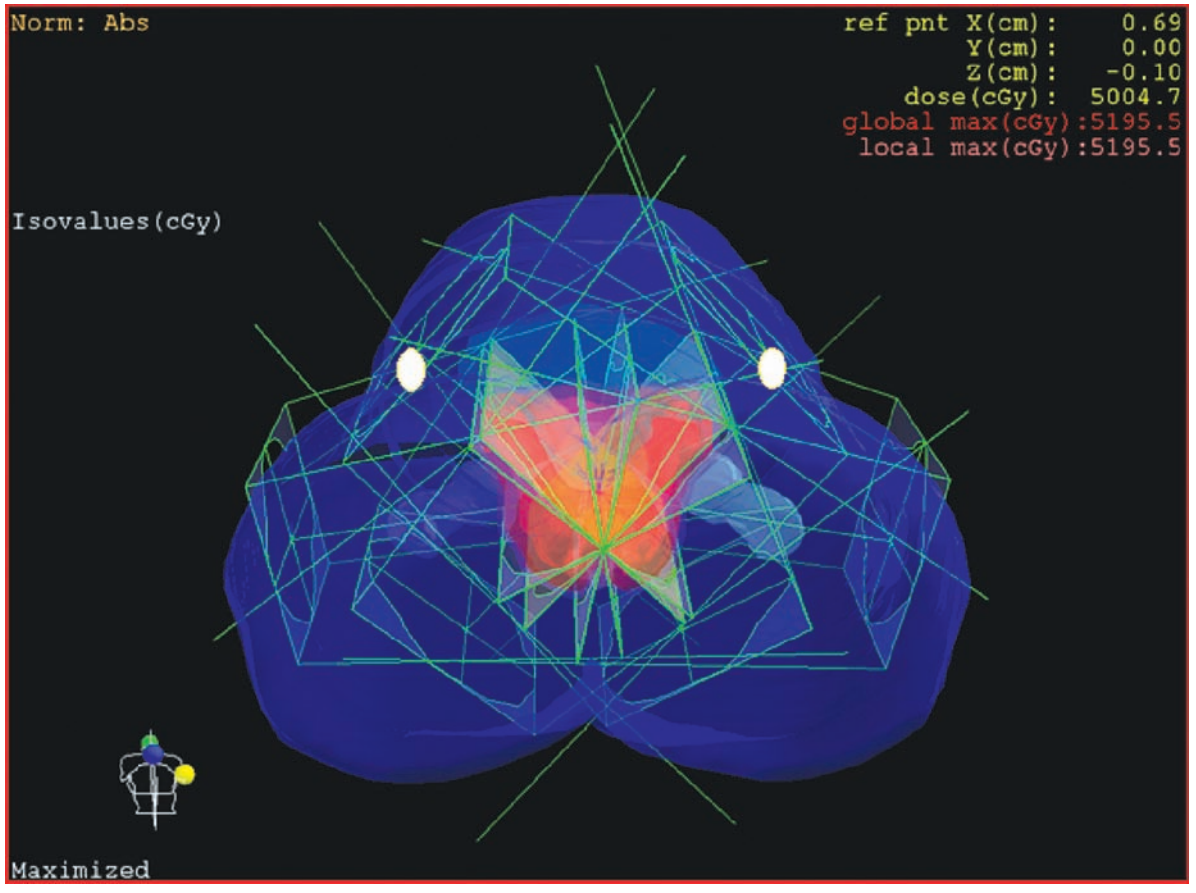



**Figure 19.3** Intensity modulated radiation therapy (IMRT).

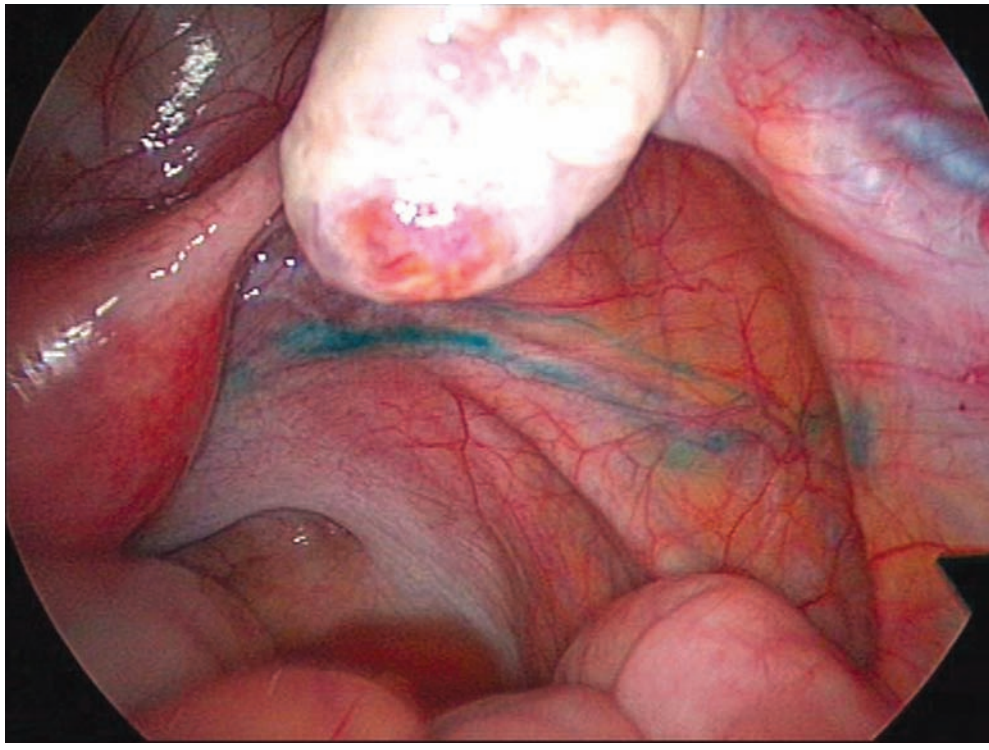


**Figure 20.1** Visualization of right pelvic lymphatic channels through laparoscopy after Patent Blue® injection in the cervix.

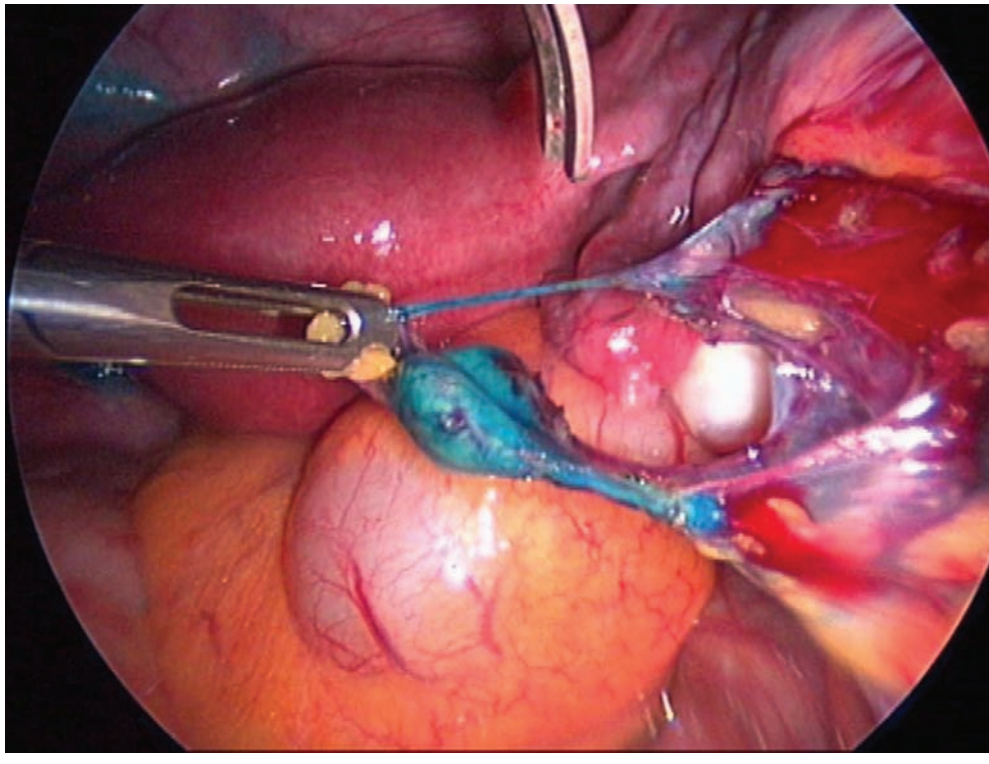


**Figure 20.2** Dissection of a right pelvic sentinel node.

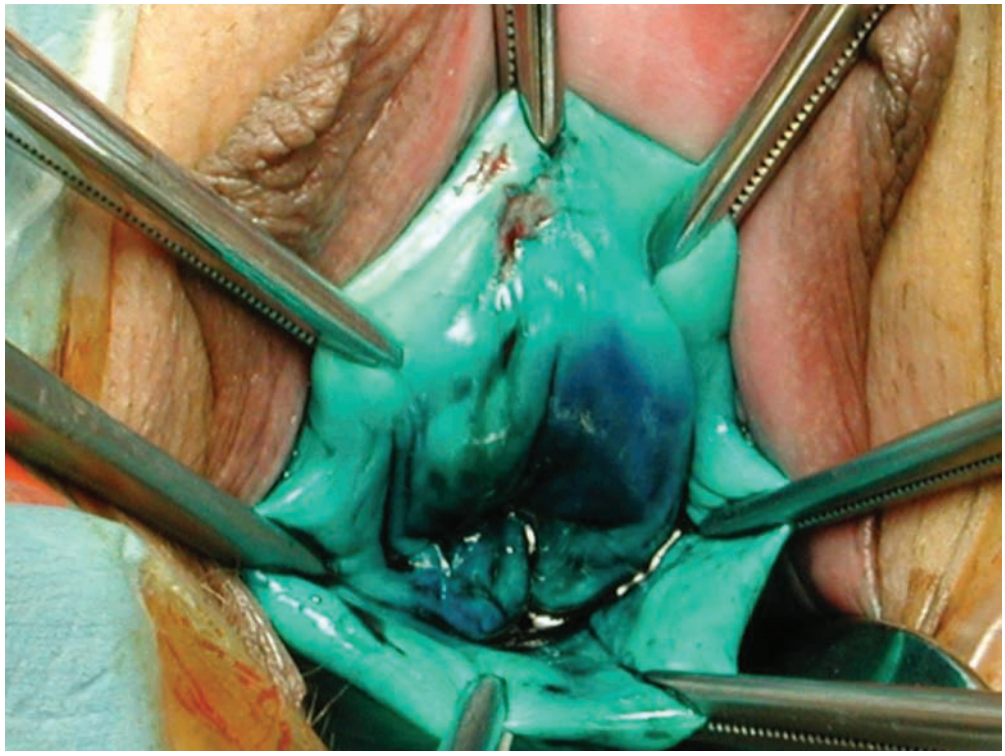


**Figure 20.3** Performance of the vaginal cuff.

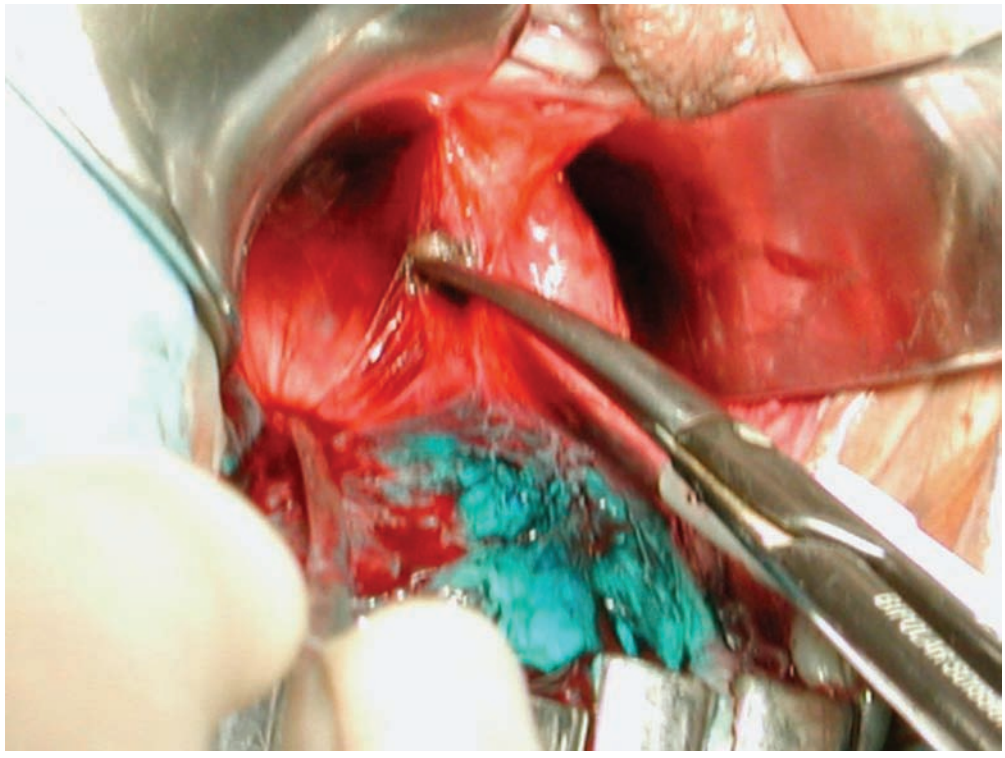


**Figure 20.4** Dissection of bladder pillars and identification of the left ureter.

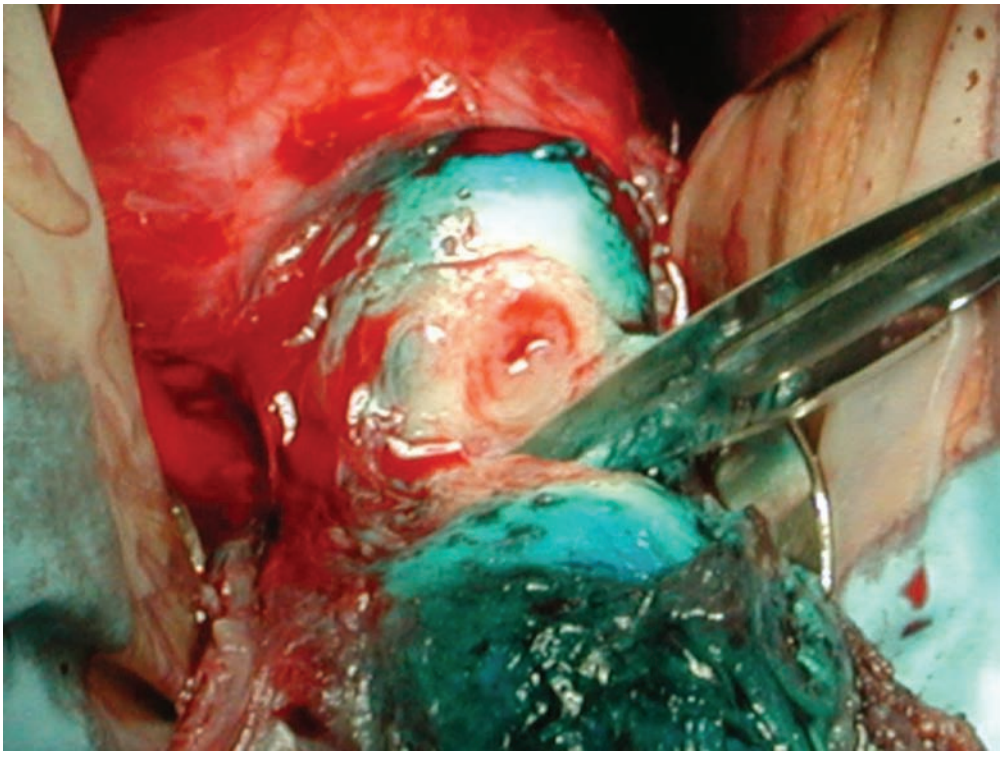


**Figure 20.6** Section of the operative specimen at the level of the uterine isthmus.

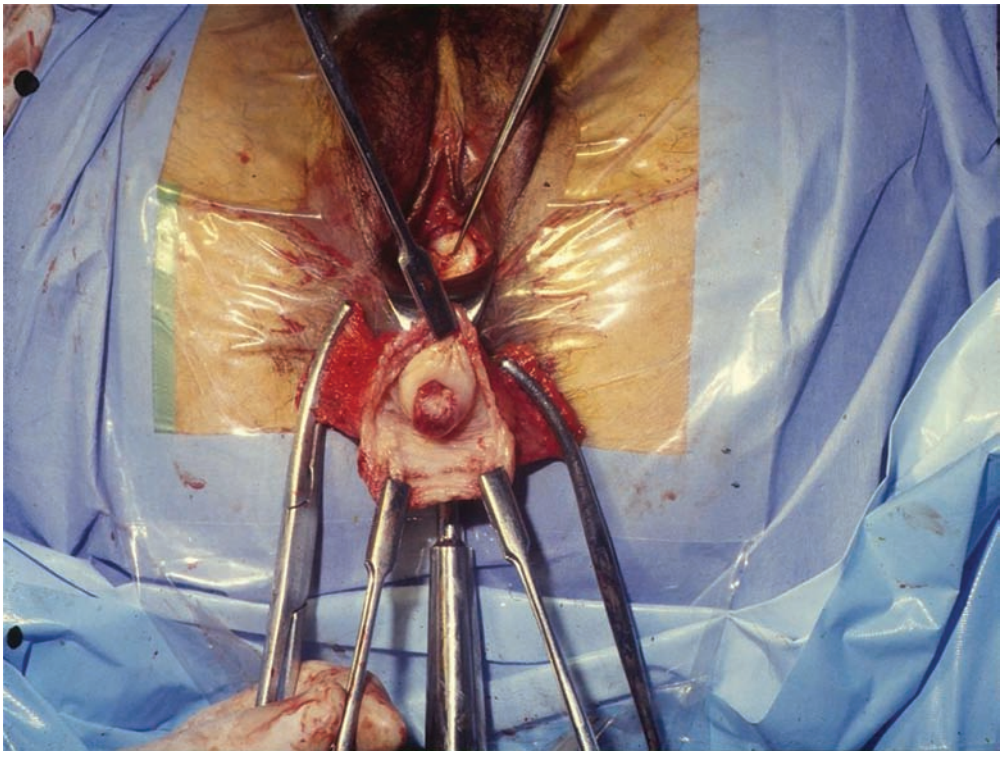


**Figure 20.8** Operative specimen of a radical trachelectomy showing the vaginal cuff and the proximal parametrial resection.

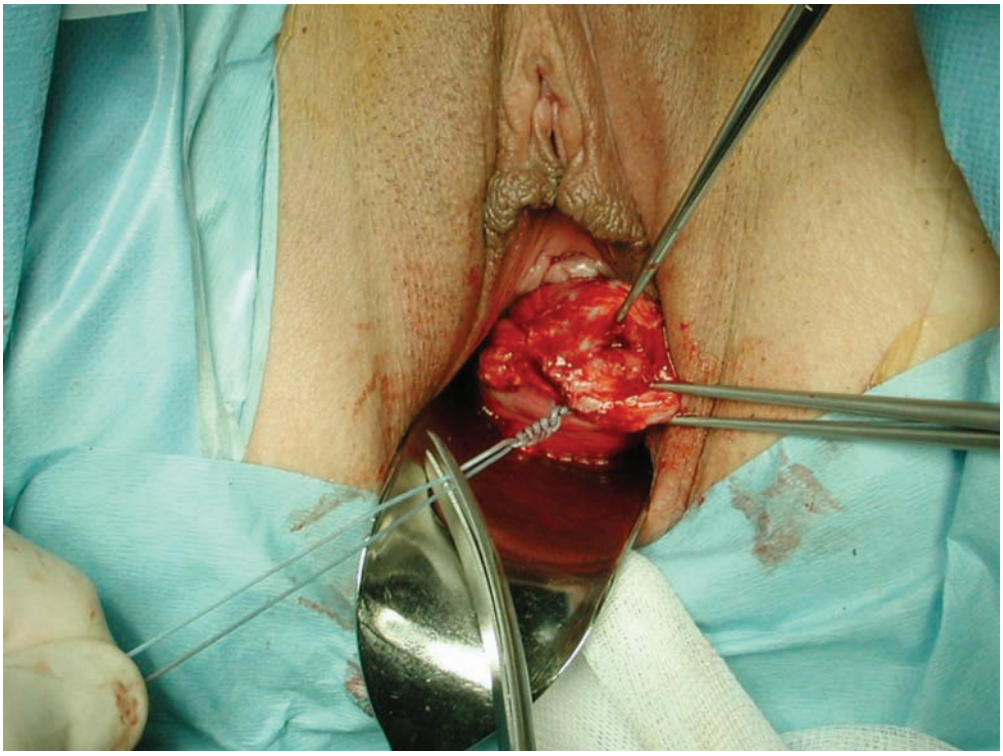


**Figure 20.9** Set up of the isthmic cerclage.

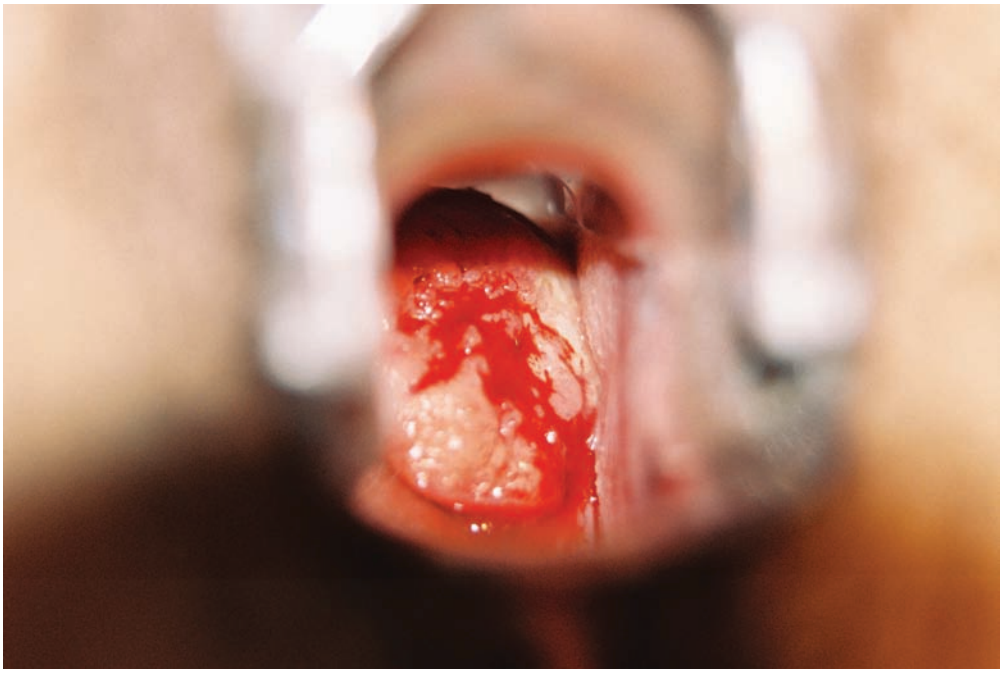


**Figure 20.10** Pre-treatment epidermoid cervical carcinoma stage IB2 (45 mm) in a 25-year-old young woman, neoadjuvant chemotherapy is planned.

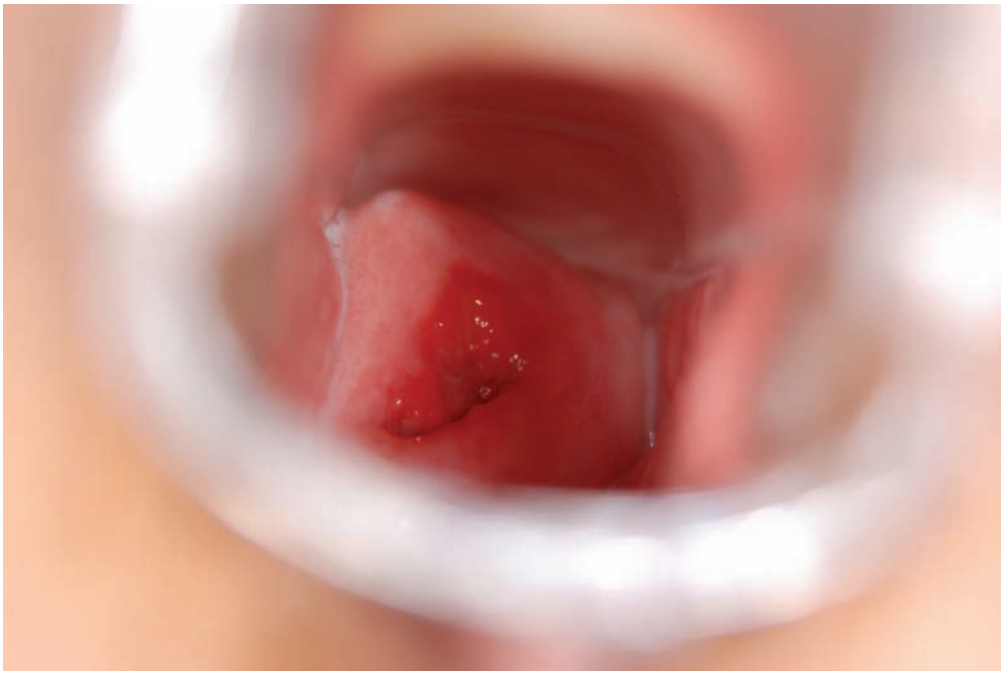


**Figure 20.11** Same patient after four courses of neoadjuvant chemotherapy: complete regression of the lesion.

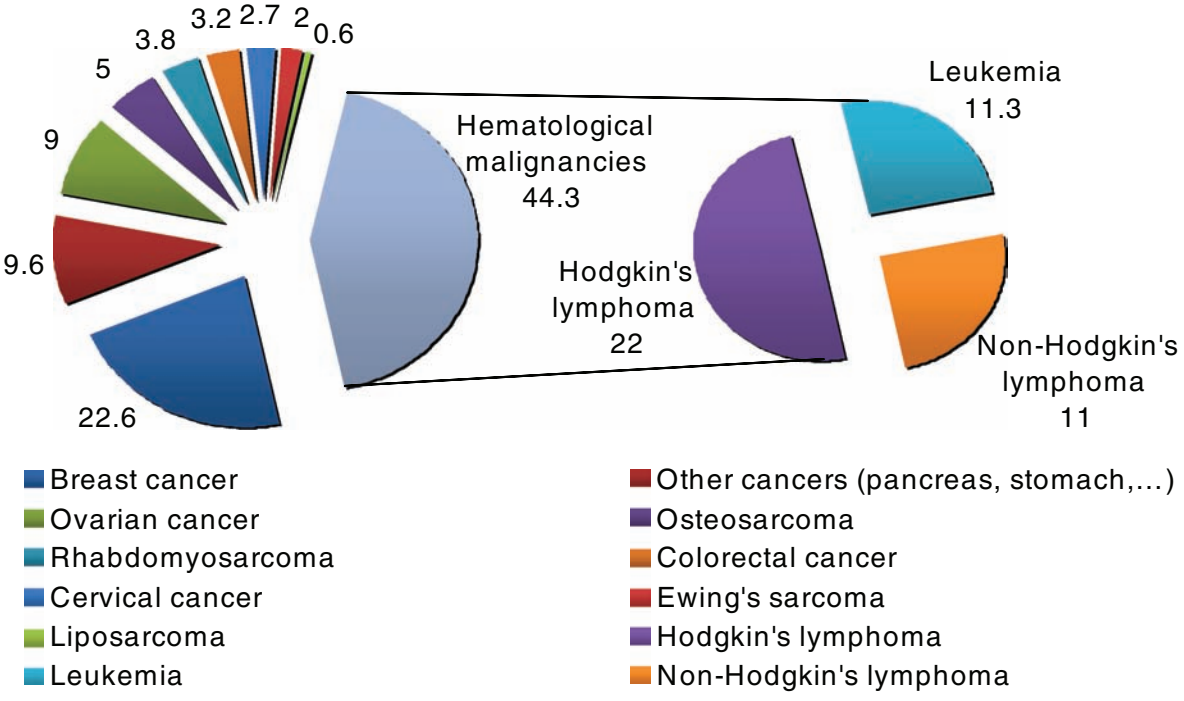



**Figure 25.1** Indications for ovarian tissue cryopreservation in case of malignant disease at Saint Luc's University Hospital, Brussels, Belguim in 2008.

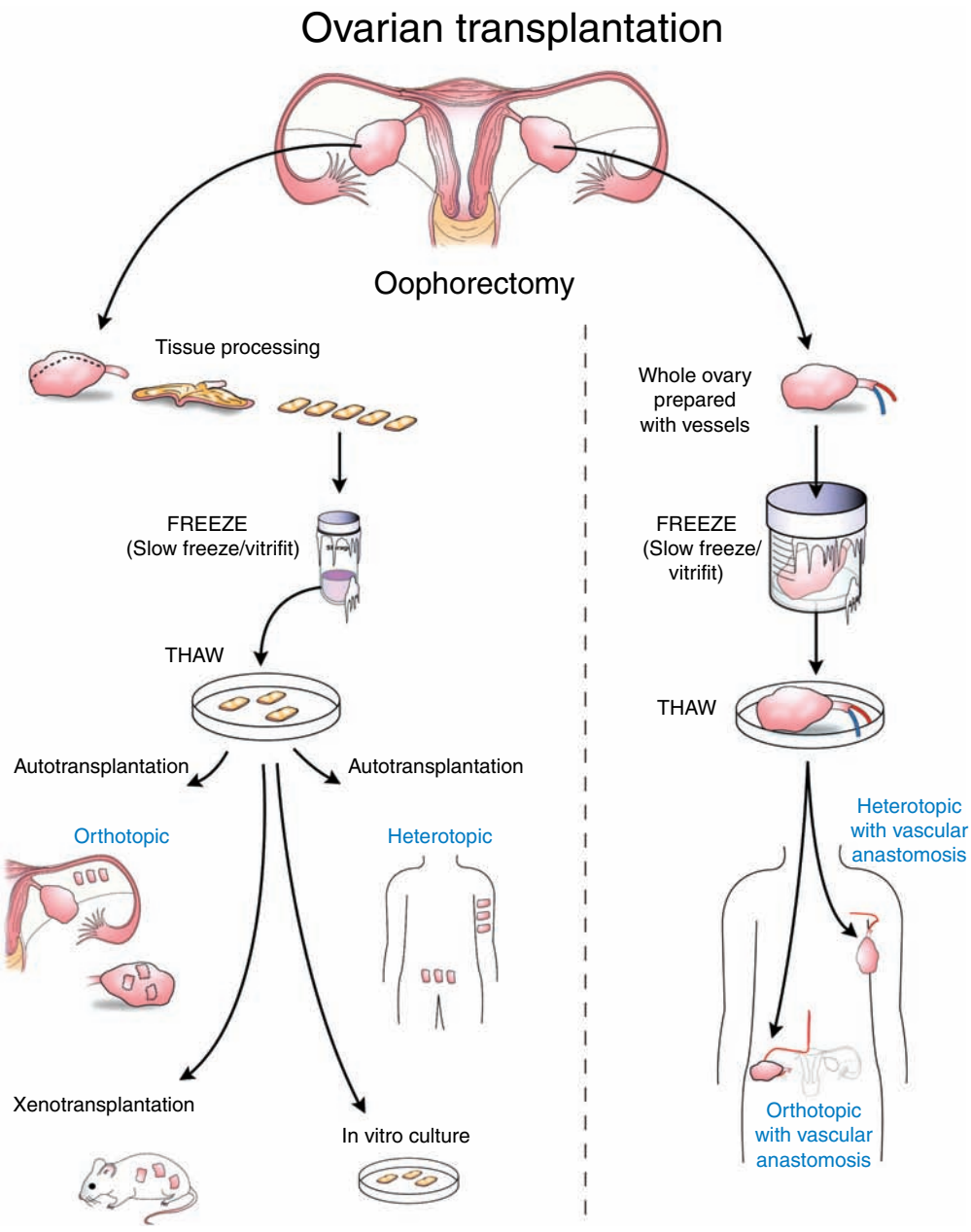



**Figure 27.4** Theoretical strategies for oocyte maturation in cryopreserved ovarian tissue and intact ovary. To date, the only strategy that produced live births is orthotopic autotransplantation of ovarian tissue.

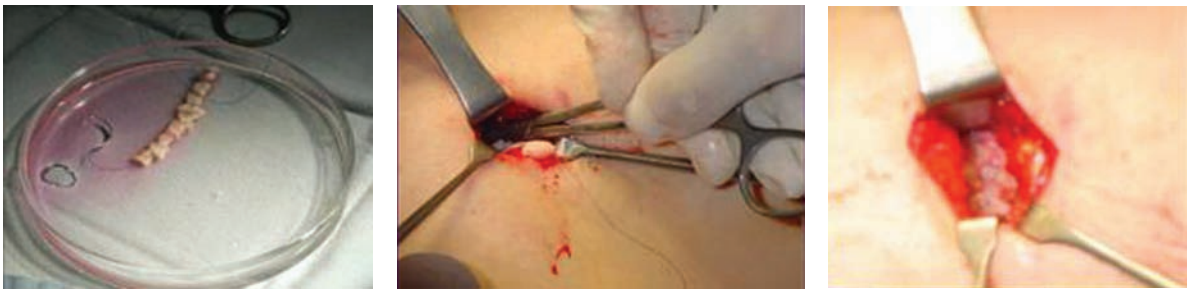


**Figure 27.5** The process of heterotopic transplantation of frozen–thawed human ovarian tissue to the space between the rectus muscle and the rectus sheath. Reproduced with permission from Elsevier.

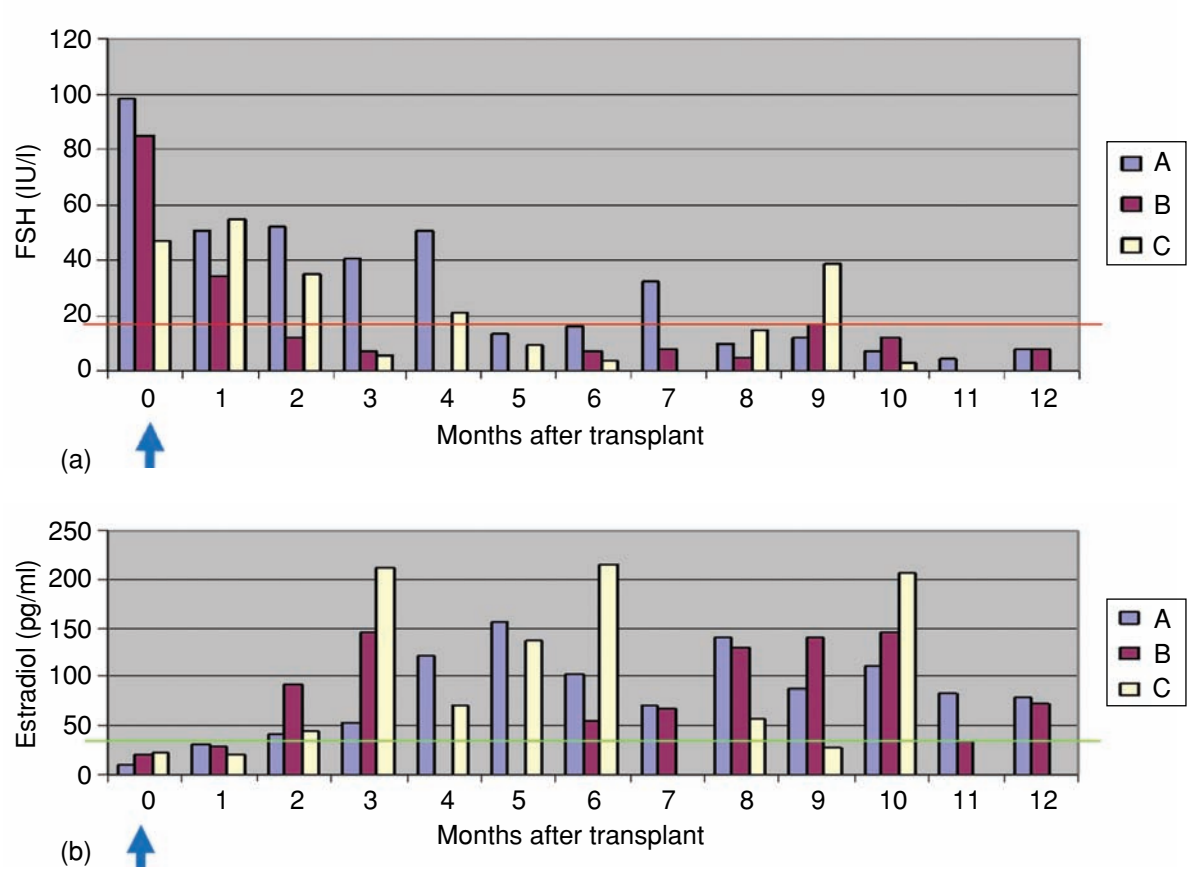



**Figure 27.6** Monthly follicle stimulating hormone (FSH) (a) and estradiol levels (b) after the second transplantation of ovarian tissue in three cancer patients (A–C). Estradiol production from ovarian grafts was noticed 2 months after transplantation, and serum FSH levels decreased below10 mIU/ml in 2 patients 3 months after transplantation. Reproduced with permission from Elsevier.

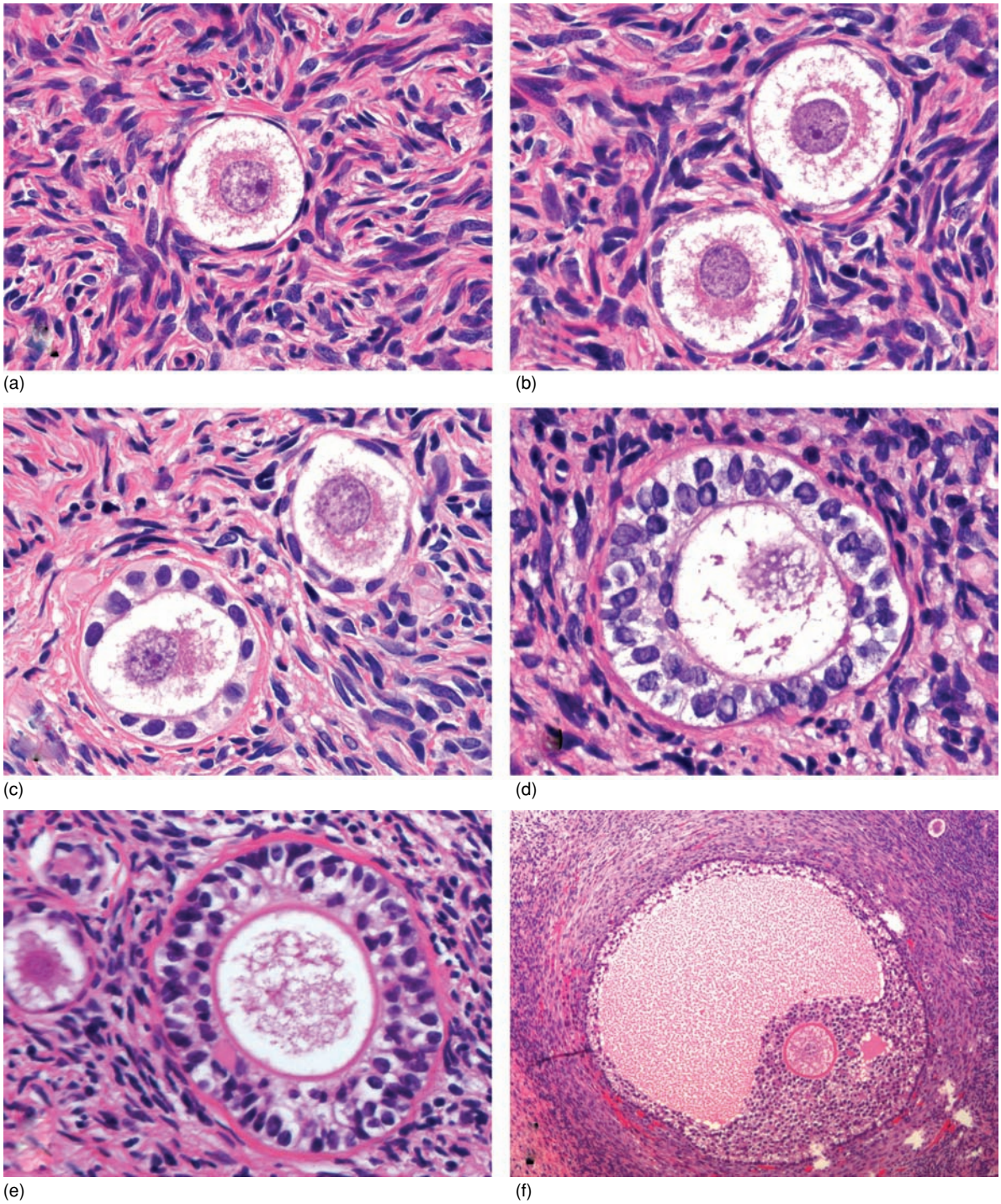
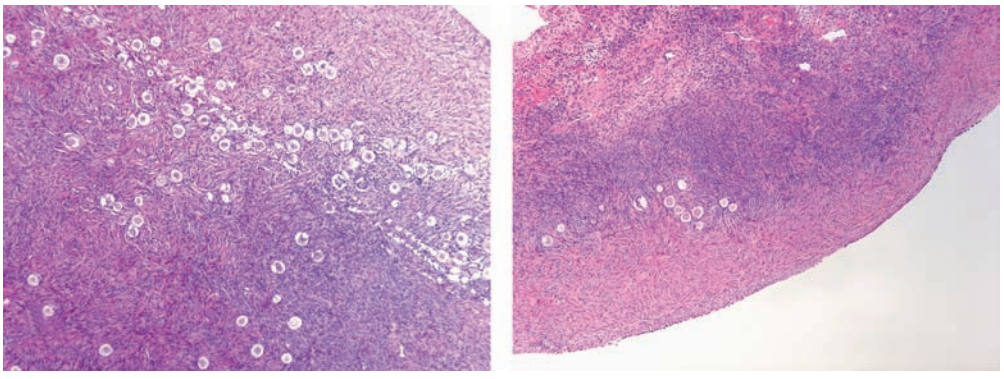
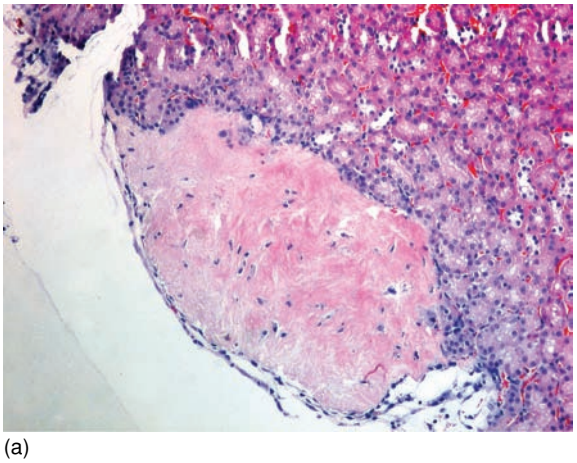



**Figure 28.1** Types of follicles observed in human ovarian tissue: (a) primordial; (b) two intermediary primordial; (c) an intermediary primordial (top) and primary (bottom); (d) proliferating; (e) secondary and an intermediary primordial; (f) an antral and a primordial (top right corner).

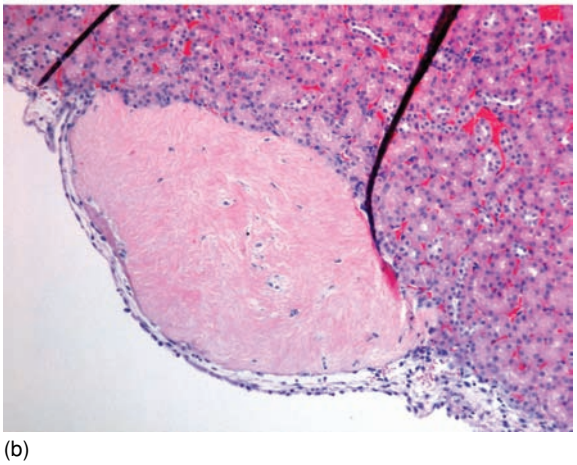


(a) (b)

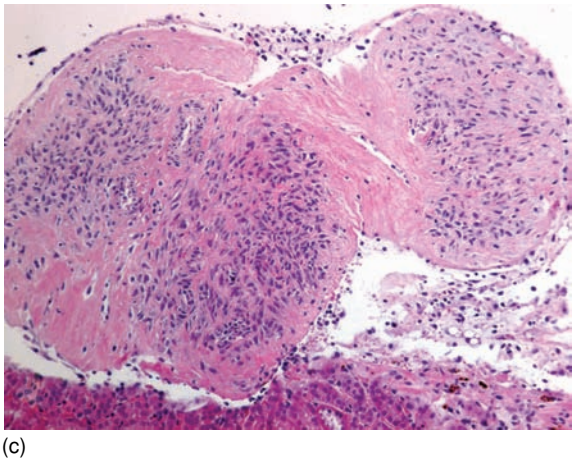
**Figure 28.2** Ovarian cortex from 2 patients: (a) an 18-year-old patient with abundant primordial follicles throughout the cortex; and (b) a 34-year-old patient with a cluster of primordial follicles.



(a)

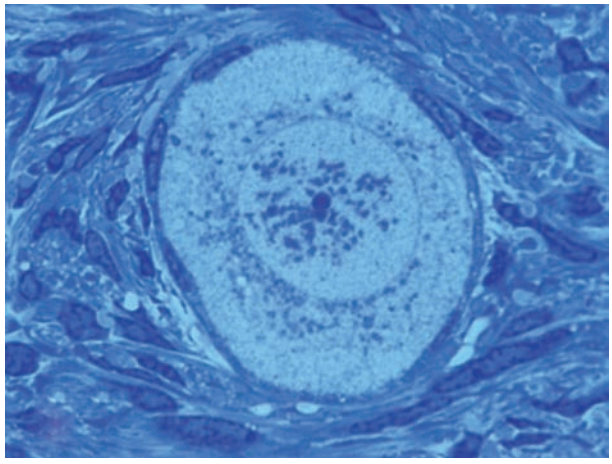


(b)

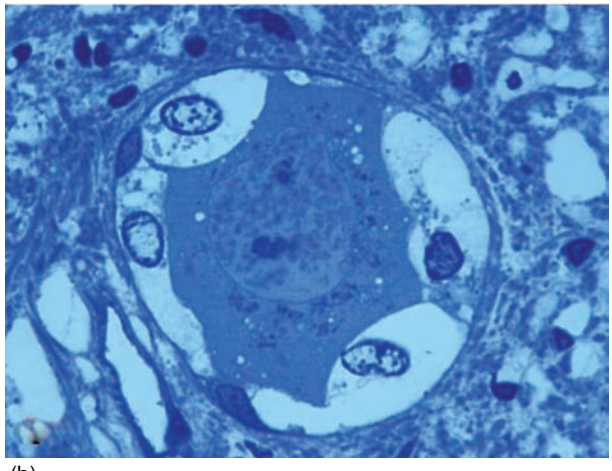


(c)

**Figure 28.3** Fresh ovarian tissue exposed to different temperatures for 2 h prior to xenografting under the kidney capsule in immunodeficient mice and subsequently examined 2 days after grafting. (a) Tissue in Quinns Hepes modified human tubal fluid (HTF) at 4°C. (b) Histidine–tryptophan–ketoglutarade solution (HTK) medium at 4°C. (c) Quinns Hepes modified HTF at 22°C. (a) and (b) magnification ×10, (c) ×5.



(a)



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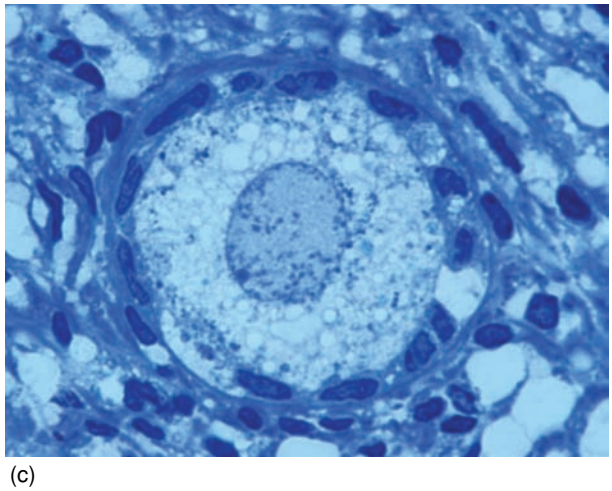


(c)

**Figure 28.4** Primordial follicles present in ovarian tissue following cryopreservation: (a) non-cryopreserved; (b) dehydrated using 1.5 M propanediol (PROH) and 0.2 M sucrose; (c) dehydrated using 1.5 M PROH and 0.1 M sucrose.

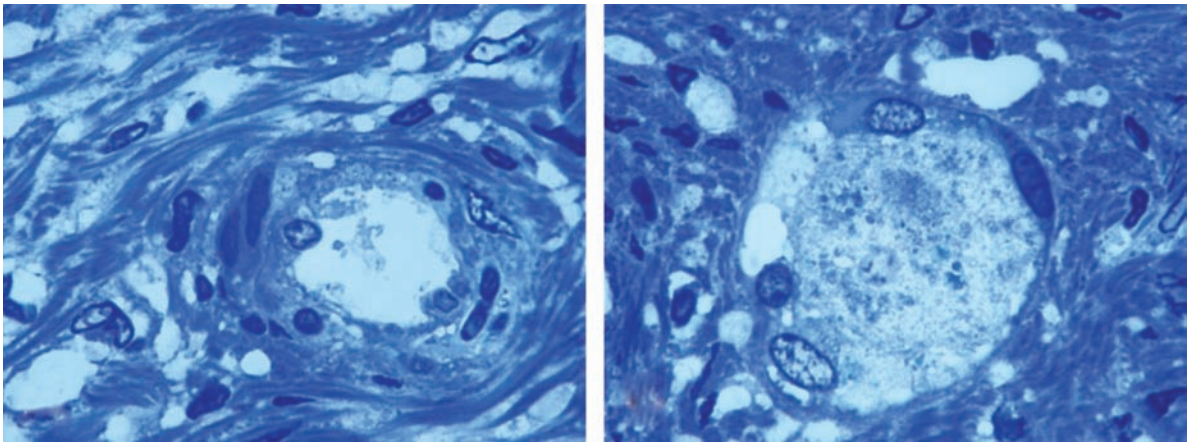



**Figure 28.5** Ovarian tissue cryopreserved using: (a) a rapid cooling rate showing remnants of a primordial follicle; and (b) an intermediate rate of cooling showing a primordial follicle containing lysed pre-granulosa cells and oocyte.

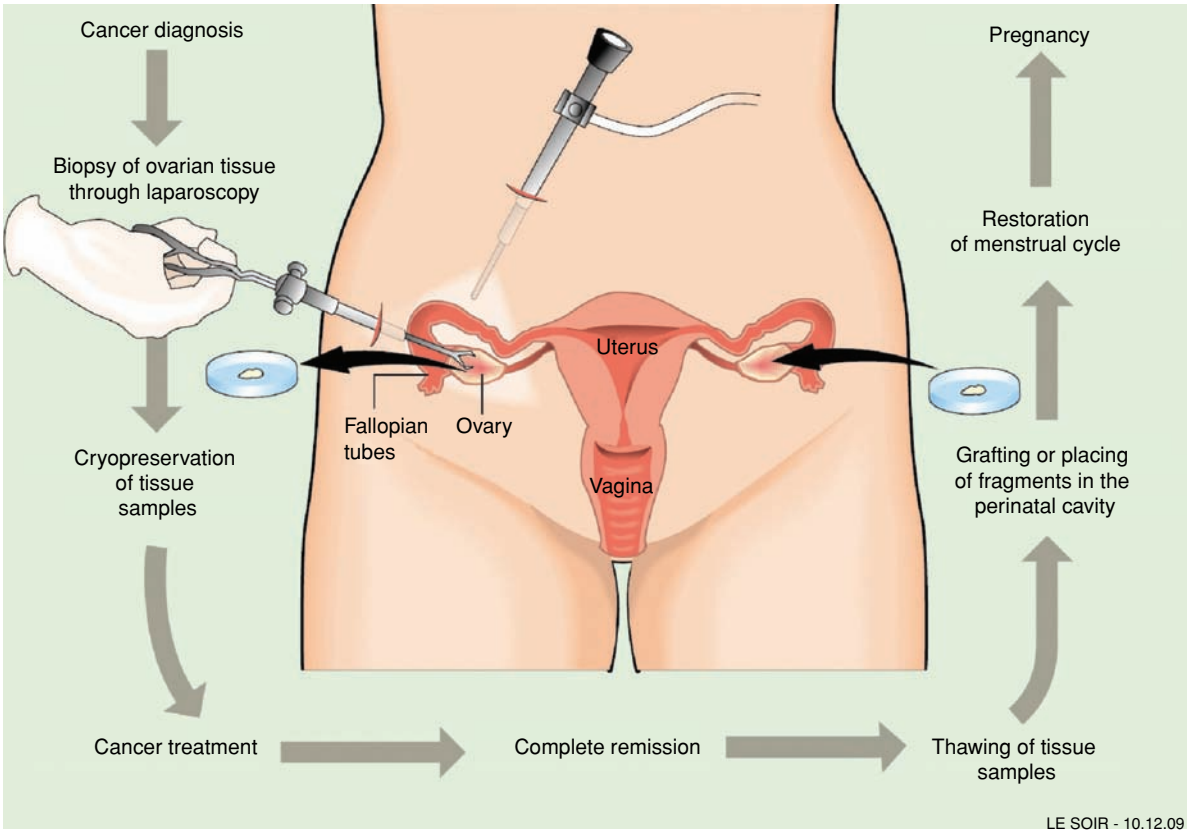



**Figure 29.1** Illustration describing the different steps from biopsy to re-implantation and pregnancy.

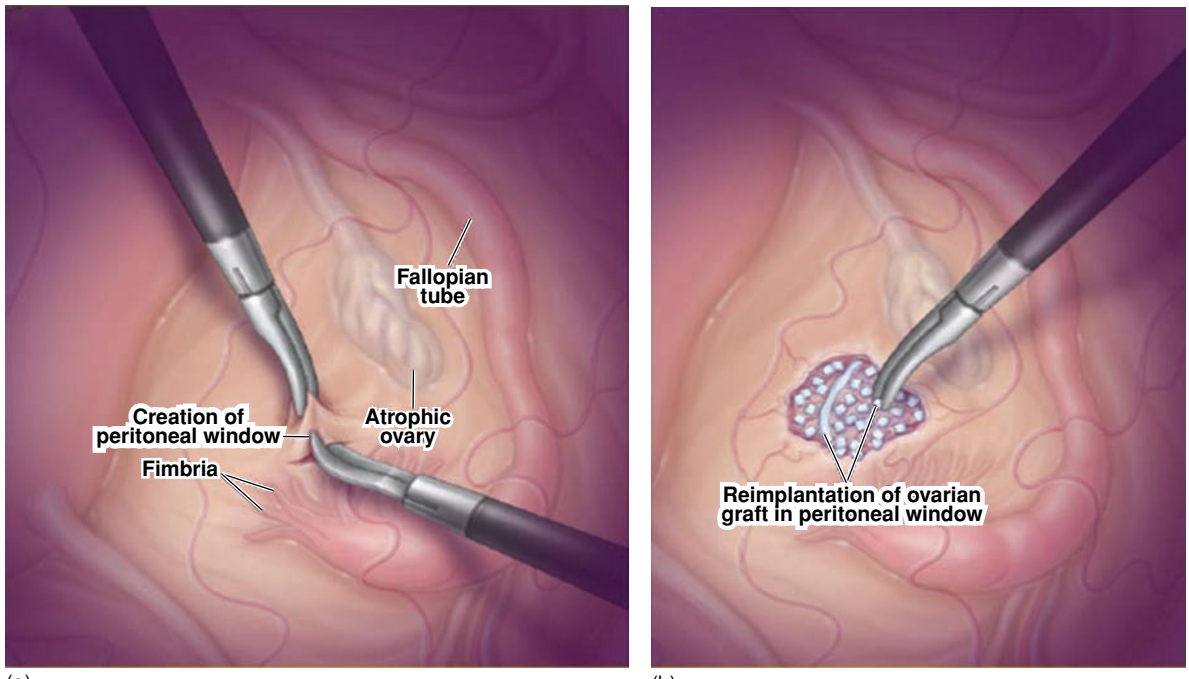



**Figure 29.2** (a) Creation of a peritoneal window close to the atrophic ovary. (b) Transplantation of ovarian cortex pieces into the peritoneal window.

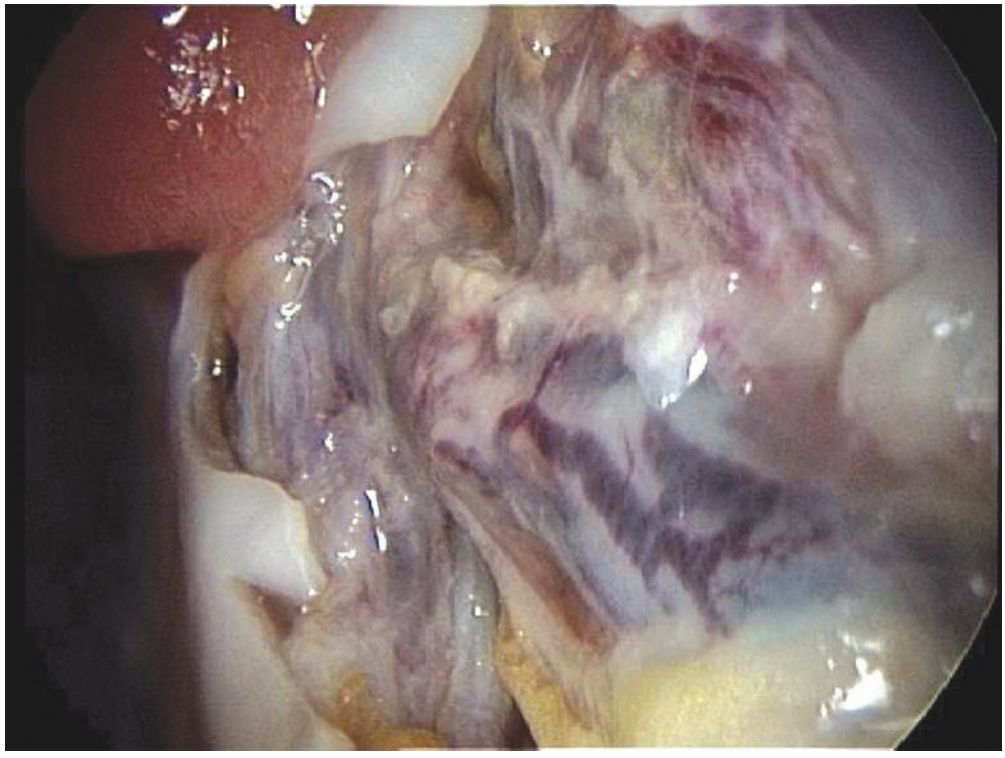
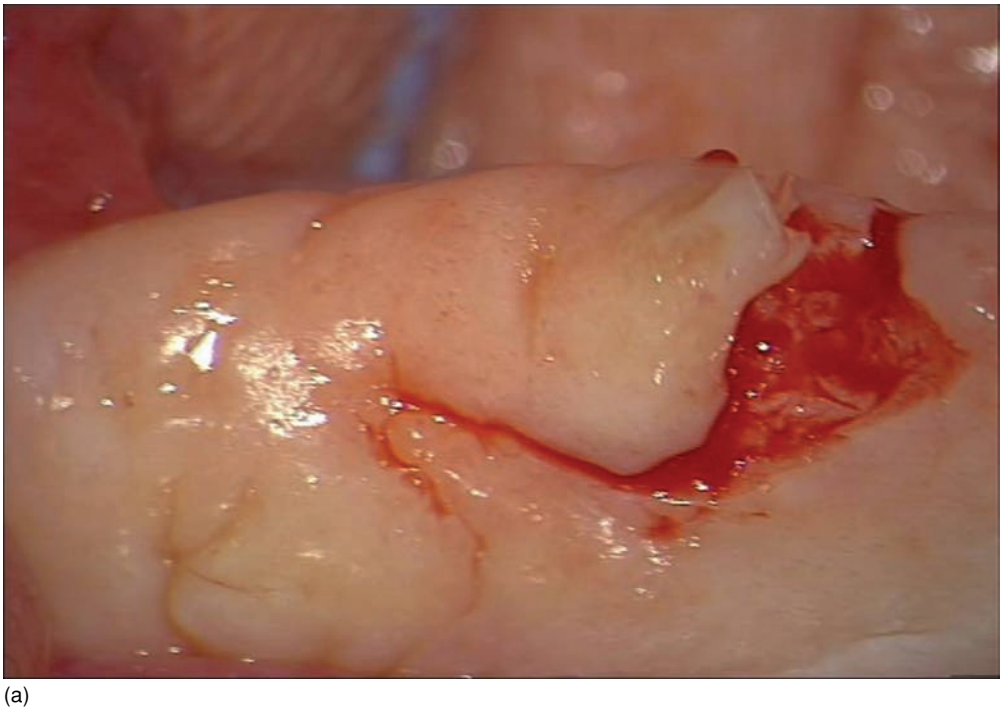
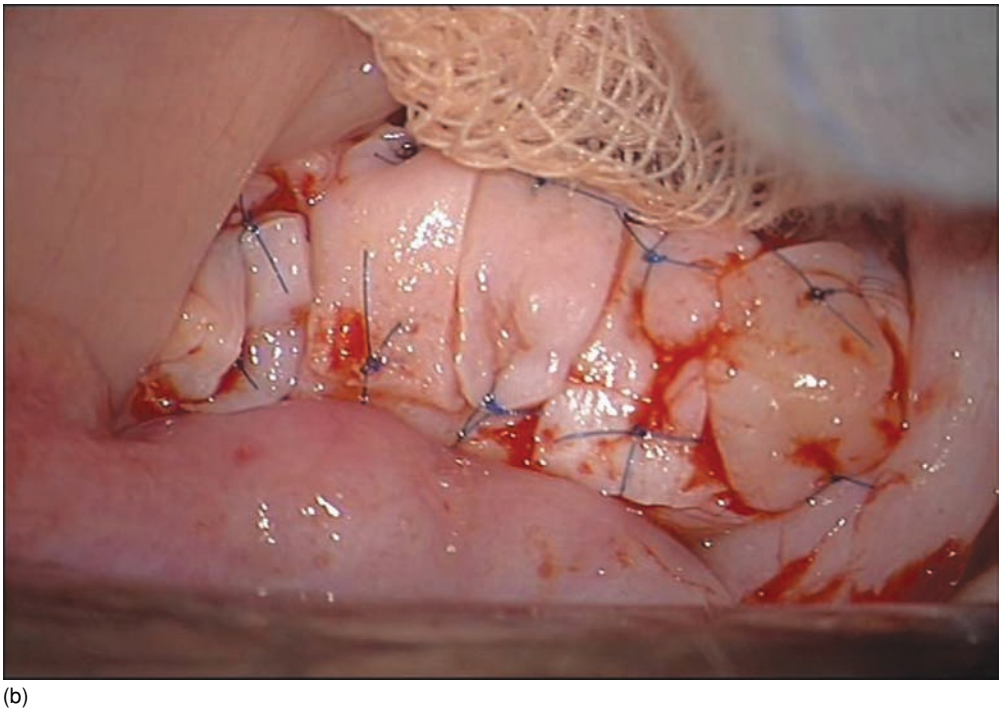


**Figure 29.3** An important vascular network is observed 7 days after the creation of the peritoneal window.



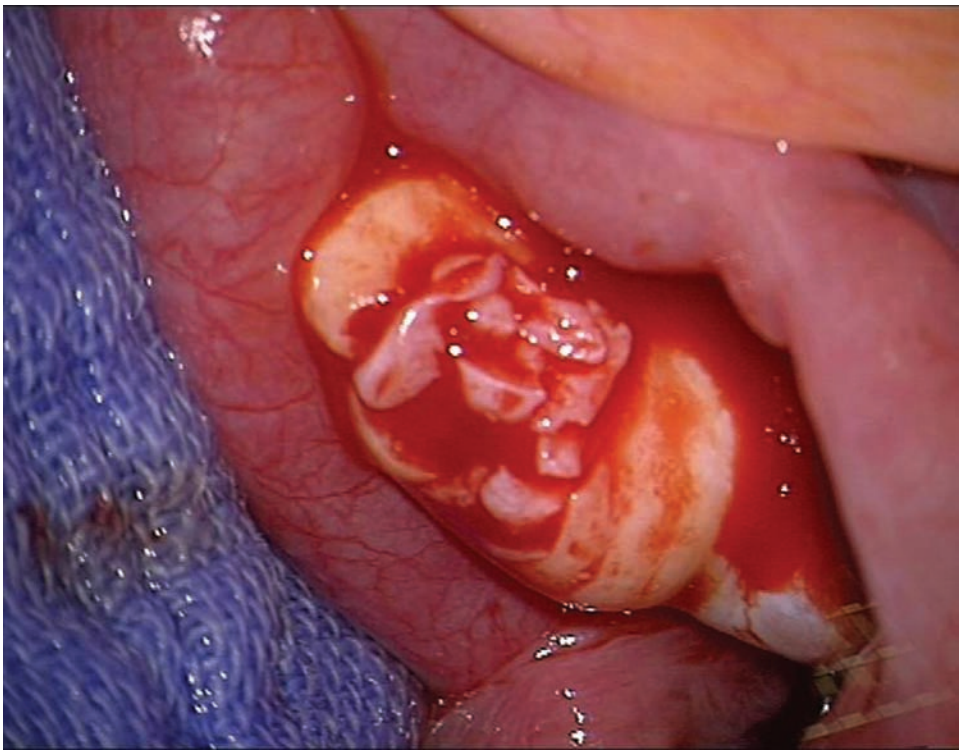
(a)

**Figure 29.4** (a) Decortication of the ovarian cortex from the remaining ovary.

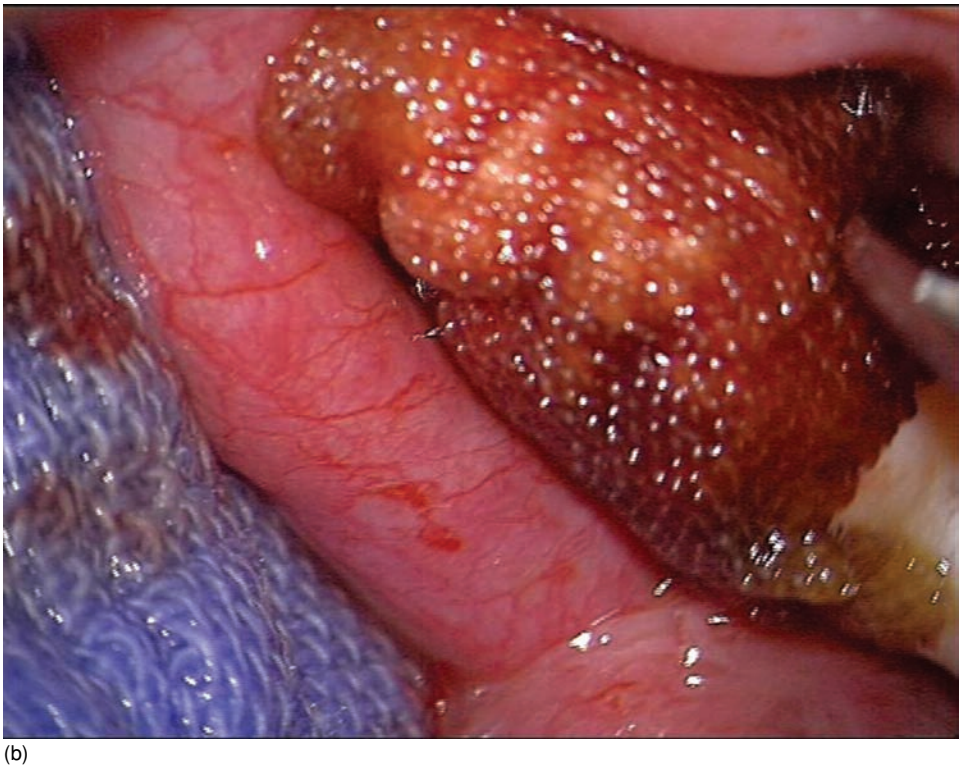


(b)

(b) Suture of the cryopreserved thawed cortical strips on the ovarian medulla.

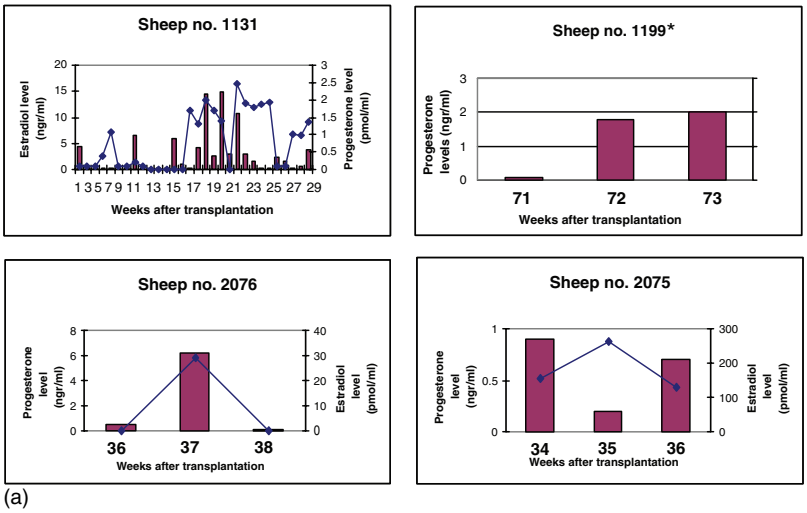


(a)

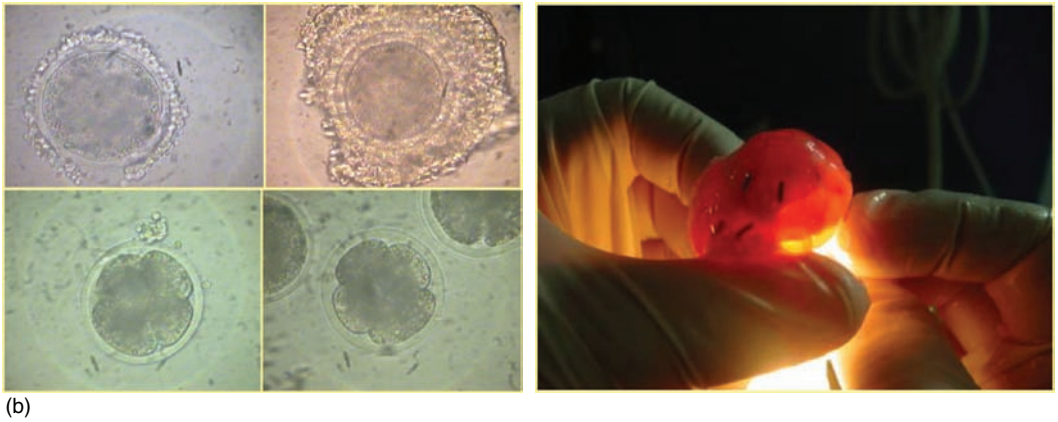


(b)

**Figure 29.5** (a) After decortication, the cryopreserved–thawed cortical cubes are placed on the ovarian medulla. (b) The ovarian cubes are covered with Interceed®.




(a)



(b)

**Figure 30.1** Endocrine function and fertility after cryopreservation and re-transplant of intact sheep ovaries at 2 and 6 years post-transplantation. (a) Hormonal levels 2 years post-transplantation [50]. (b) Follicles by transillumination 6 years post-transplantation [51].

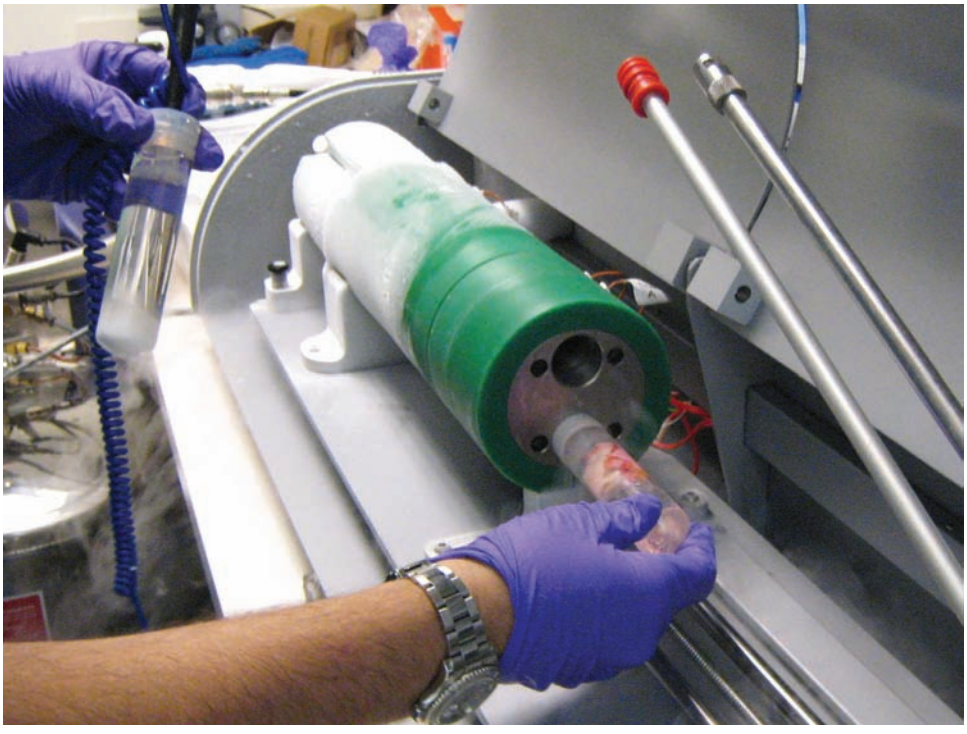


**Figure 30.2** Whole human ovary cryopreservation with the vascular pedicle utilizing the same Multi-Thermal-Gradient device and slow cooling, rapid thawing protocol as described in the text.

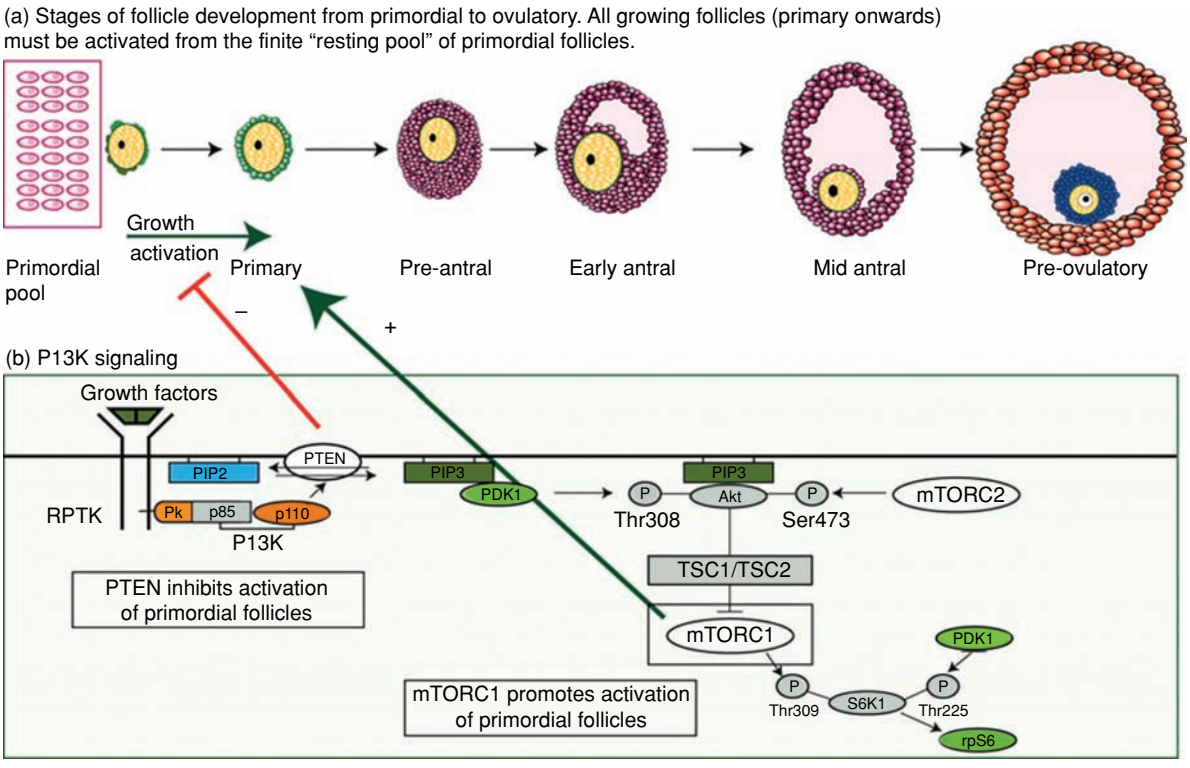



**Figure 33.1** (a) Digrammatic representation of follicle growth from the non-proliferating pool of primordial follicles. Primordial follicles are continuously activated into the growing population where they become primary follicles consisting of an oocye arrested at the dictyate stage of prophasel of meiosis (yellow) surrounded by granulosa cells (green). Primary follicles undergo oocyte growth and granulosa cell proliferation and differentiation (purple) when they form an antral cavity. Antral follicles continue to grow and granulosa cells differentiate into two subpopulations of cells: (1) cumulus surrounding the oocyte (blue); and (2) mural lining the wall of the follicle (orange). Exact timings for this developmental sequence to occur in humans are not known but estimations suggest several months. However, it is not known whether the growth profile is continuous or whether there are "resting" phases through follicle development. (b) Simplified version of the PI3K pathway. The factors initiating this process are largely unknown but a body of evidence is emerging to show that the phosphatidylinositol-3'-kinase (PI3K-AKT) signaling pathway is a major regulator of early follicle/oocyte development and that components of this pathway are involved in controlling the rate of activation from the non-growing population of follicles. The phosphatase PTEN converts PIP3 to PIP2, which negatively regulates PI3K activity. Signaling mediated by PI3Ks converge at PDK1. PDK1 phosphorylates Akt and activates it. Akt can phosphorylate and inactivate tuberous sclerosis complex 2 (TSC2 or tuberin), which leads to the activation of mTOR complex (mTORC1). mTORC1 can phosphorylate (activate) S6K1. S6K1 subsequently phosphorylates and activates rpS6, which enhances protein translation that is needed for cell growth. mTORC1 can be inhibited pharmacologically with Rapamycin and stimulated by leucine. The manipulation of this pathway could have important clinical applications in the field of fertility preservation.

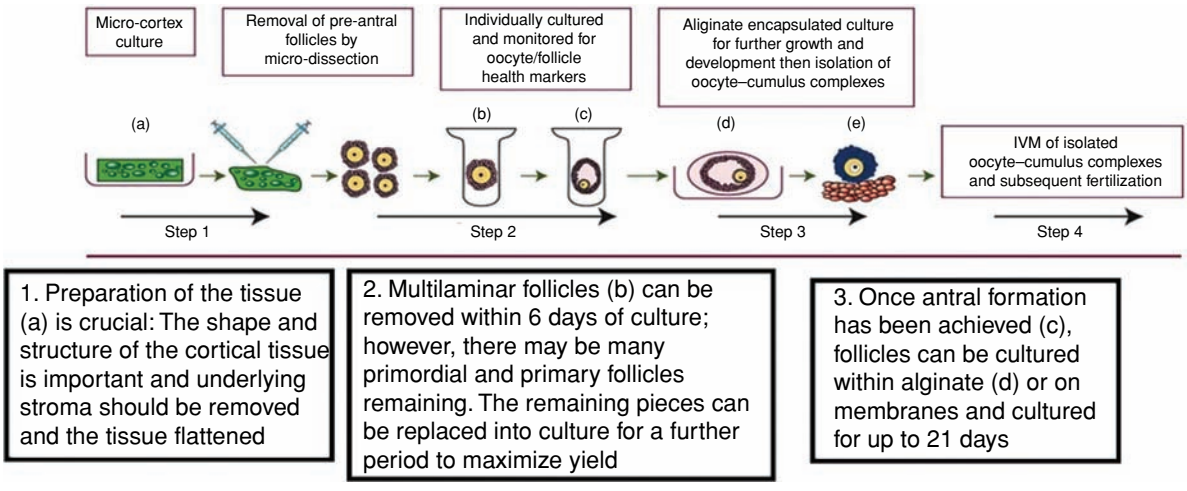



**Figure 33.2** Proposed multi-step culture system for activation of human primordial follicles and subsequent follicle/oocyte development. The stages required for a multi-step culture system are as follows. Activation of primordial follicles within cortical strips (a). Removal of all growing follicles and most of the underlying stromal cells increases the rate of activation [6]. Flattened strips are cultured free floating in medium containing human serum albumin (HSA), ascorbic acid and basal levels of follicle stimulating hormone (FSH) [6]. Once follicles have reached multilaminar stages they are isolated mechanically using needles and cultured individually Isolated follicle culture is to support development from pre-antral to antral stages (b). The addition of activin at this stage results in improved follicle development and increased antral formation (c) [6]. Follicles of similar stages that have been grown in vivo have been isolated and grown with alginate drops [7] (d), and oocytes grow to almost full size within a total of 30 days [7]. The final stages of oocyte growth and development could be achieved by culturing the oocyte and its surrounding somatic cells outwith the constraints of the large follicle (e).

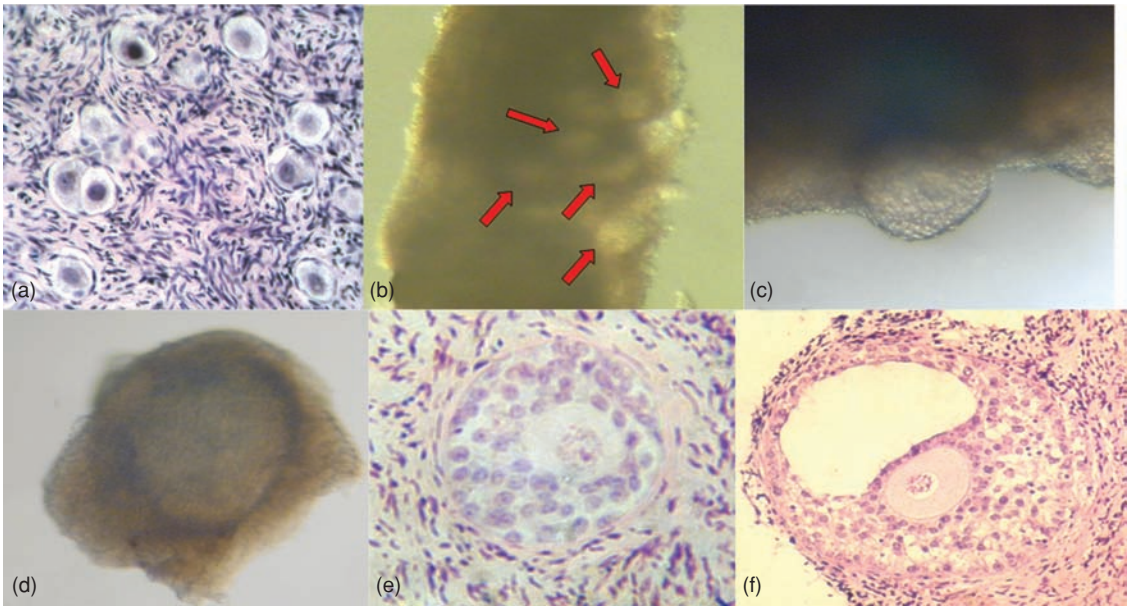



**Figure 33.3** (a) A cluster of quiescent follicles in freshly fixed human ovary. (b) After 6 days in vitro, growing follicles (↑) appear on the surface of a cultured fragment of human ovarian cortex. (c) A growing follicle protruding from the edge of a fragment of cultured human cortex. (d) Intact secondary human mechanically dissected with presumptive theca layers attached. (e) Histological image of a secondary human follicle fixed after 6 days in vitro growth within a cortical fragment. (f) Histological image of human antral follicle fixed after a total of 10 days in vitro growth.

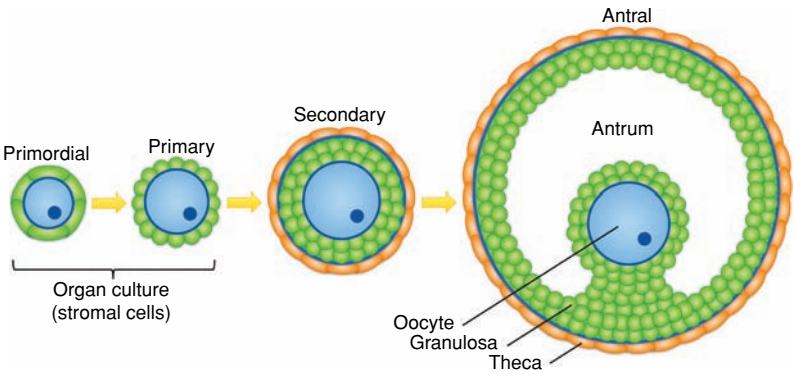



**Figure 34.1** Folliculogenesis. Primordial follicles develop to antral follicles, which are capable of producing fertilizable oocytes. Ovarian stromal cells are hypothesized to have significant roles in the activation of primordial follicles and the recruitment/differentiation of theca cells. Most follicle culture systems focus on secondary or multilayer follicles and produce antral follicles with fertilizable oocytes. Primordial and primary follicles do not activate or mature in vitro. The culture of these follicles is typically performed as an organ culture.

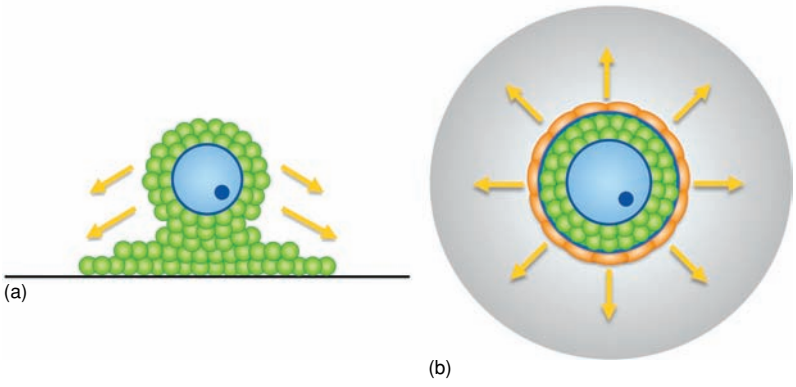



**Figure 34.2** In-vitro follicle culture systems. (a) In two-dimensional systems, follicles are cultured on flat surfaces such as tissue culture plastic (polystyrene). The unnatural geometry/mechanics of these systems disrupts cell–cell communication and causes the granulosa cells to break though the basement membrane, migrate away from the oocyte, and attach to the two-dimensional surface. Two-dimensional systems lack the ability to support large follicles for extended culture times. (b) In three-dimensional systems, follicles are cultured within biomaterial scaffolds, such as alginate. These systems maintain the natural spherical geometry and cell–cell interactions of the follicle.

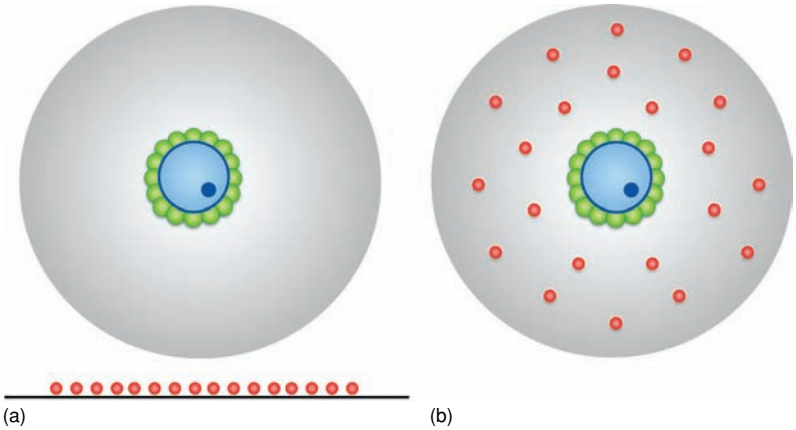



**Figure 34.3** Stromal cell co-culture approaches. (a) Stromal cells can be cultured separately on a flat surface below the encapsulated follicle. This set up allows for paracrine signaling between the follicle and the stromal cells. (b) Stromal cells can be encapsulated inside the biomaterial scaffold with the follicle. This set up allows for paracrine signaling as well as cell–cell attachment and interaction with secreted extracellular matrix proteins.

Chapter 30

# Whole ovary freezing

J. Ryan Martin, Jason G. Bromer and Pasquale Patrizio

## Introduction

Patients with cancer who desire to preserve their future reproductive potential but require immediate gonadotoxic treatments (chemo and/or radiotherapy), are left with few options for fertility preservation. These options include: (a) cryopreservation of ovarian tissues as cortical strips; (b) dual cryopreservation of both ovarian cortical tissue and cryopreservation, after in vitro maturation, of immature oocytes extracted from the small antral follicles visible within the ovarian cortex at the time of the harvest; (c) cryopreservation of one whole ovary [1–9]. Each of these options is still considered experimental (thus requiring Institutional Review Board approval and patient's informed consent).

Ovarian cryopreservation and transplantation [10–11], either as heterotopic or orthotopic allografts, has shown some reproductive success [12–14]. At the time of writing, a total of seven live births from re-transplantation of ovarian cortical tissue to an orthotopic location have been reported [15–19], while four more have been announced at a meeting, but not yet published. Typically, it takes about 4–5 months for resumption of endocrine function as evidenced by menses or serological hormonal evaluation. However, the re-transplanted cortical pieces only retain ovarian function for a short time and almost all ceased to function by 3 years [6, 20, 21].

There are several reasons to explain this transient return of ovarian function followed by the rapid decline. One reason is that the amount of cryopreserved/thawed cortical tissue re-transplanted during a graft is limited. Another reason for the short longevity is that the cortical tissue is grafted without a vascular anastomosis and is, therefore, completely dependent for its survival on the development of a new vasculature; a process which requires at least a week [22, 23]. By the time neovascularization occurs, the grafts will have already sustained significant ischemic damage resulting in massive loss of primordial follicles [13, 24–27], ultimately responsible for the limited functional life span of the graft [28, 29]. This process was illustrated in a sheep model that found that 60–70% of follicles were lost at transplantation, but only 7% of the loss was dependent upon the cyropreservation procedure itself [30].

Cryopreservation of the whole ovary with its intact pedicle and vascular supply has recently been proposed as an experimental strategy that could potentially overcome the problem of ischemic damage; in these instances a successful re-anastomosis will provide immediate reperfusion so, theoretically, the ovary should maintain long-term endocrine and reproductive functions [2, 24, 31–33].

This chapter summarizes the technical challenges that had to be overcome for the freezing/thawing of intact ovaries with animal experiments first and then with humans.

## Challenges of whole ovary cryopreservation

Two main problems with whole organ cryopreservation and re-transplantation have caused technical difficulties: the first related to the feasibility of executing a perfect vascular re-anastomosis of the whole organ with re-establishment of a prompt vascular flow; the second was related to the development of a successful cryopreservation protocol.

Experiments with whole fresh ovary re-transplants, have proved that the re-anastomosis of the ovarian pedicle is technically possible in rabbits

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[34], sheep [35–37], dogs [38], monkeys [39] and also in humans [24, 28, 40–42]. Wang *et al.* demonstrated that cryopreservation and subsequent re-transplantation of the reproductive system en bloc could be performed in rats [33]. To date, two cases of human re-transplant of fresh whole ovaries and one live birth have been reported [43]; however, no cases of frozen/thawed whole ovary re-transplants have been performed yet.

The second challenge, i.e. creating a successful cryopreservation protocol for large-sized intact ovaries, has proved more problematic due to: (a) heat and mass transfer problems; (b) the physical constraints related to the heat transfer between the core and periphery of a large organ; and (c) the establishment of adequate stromal and cortical diffusion of the cryoprotectants [44, 45] to prevent the formation of intravascular ice [24]. Large amounts of ice crystals are a major destructive force for cells and are known to be mechanically disruptive. Inadequate perfusion of the cryoprotectants into the vascular compartment may lead to intravascular ice formation with subsequent vascular injury and irreversible endothelial disruption [46]. Slow cooling methods were developed to allow a slow enough process to dehydrate cells in order to prevent intracellular crystallization but, at the same time, fast enough to minimize osmotic stress to cells.

## Whole ovary freezing in rat model

Wang *et al.* investigated cryopreservation after immersion in liquid nitrogen and re-transplantation with microscopic re-anastomosis of whole rat ovaries [33]. In this landmark study, the authors used adult female rats and removed the right ovary and the upper segment of the uterus en bloc, with the ovarian vessels dissected to create short cuffs of aorta and vena cava. Seven dissections were perfused for 30 min at 0.35 ml/min with M2 medium containing 0.1 M fructose and increasing concentrations of dimethyl sulfoxide (DMSO). The treated organs were then cooled slowly in Cryovials and, after overnight storage in liquid nitrogen, were rapidly thawed and the cryoprotectant was removed.

After re-transplantation, four of the seven rats had subsequent follicular development, with corpora lutea indicating recent ovulation, and one animal achieved pregnancy. Importantly, tubal and uterine morphology and architecture were indistinguishable from non-operated controls. However, these rats had higher serum follicle stimulating hormone (FSH) levels, fewer follicles and lower estradiol levels and uterine weights than controls, indicating that the freezing had compromised the ovarian function.

Yin *et al.* continued this work using a similar model to investigate the long-term longevity of ovarian grafts, as well as to assess the effect of ischemia after cryopreservation for 24 h prior to transplant [47]. Graft survival with endocrine function was seen at 2 months, and ovulatory response to FSH was seen at 4 months, suggesting good graft survival after vascular anastomosis. These grafts however had fewer surviving follicles than the controls, emphasizing that even minimal ischemia time significantly reduces the follicular pool.

## Whole ovary freezing in sheep model

To improve the technique of freeze–thaw and transplantation of whole ovaries, adult female sheep have become the preferred animal model to study both slow cooling and vitrification methods. Sheep have ovaries that are similar in size to humans and are therefore ideal animal models. Despite human ovaries having different vascular pedicle anatomy, the sheep ovaries have dense fibrous stroma and a relatively high primordial follicles density in the cortex similar to human ovaries [48].

### Slow cooling

In 2002, Jeremias *et al.* attempted orthotopic transplantation of a whole ovary in an adult sheep by anastomosis of the vascular pedicle [36]. After bilateral laparoscopic oophorectomy, ovaries were autotransplanted into the abdominal wall, and microsurgical vascular anastomosis of the ovarian to the inferior epigastric vessels was performed. After noting promising resumption of endocrine function post-transplant and a high follicular count, the authors concluded that, in conjunction with an improved protocol for cryopreservation, "ovarian autotransplantation with vascular anastomosis may be superior to ovarian tissue banking and grafting techniques" [36].

The same team also performed also one of the first transplants of an intact frozen–thawed ovary via microvascular anastomosis [44]. After laparoscopic dissection, the whole ovaries were immediately perfused with heparin, followed by perfusion and immersion in a bath containing Leibovitz L-15 medium, 10% fetal bovine serum (FBS) and 1.5 M DMSO. Ovaries were perfused via the ovarian artery with the

cryoprotectant solution at a rate of 1.3 ml/min. After perfusion, the ovaries were transferred into a Cryovial and cooling began at 4°C and at 2°C/min until ice nucleation was induced at –7°C. The temperature was then reduced by 2°C/min until –35°C and, subsequently, by 25°C/min until –140°C after which the Cryovials were plunged into liquid nitrogen. Thawing occurred 1 week later, and was achieved by first plunging and swirling Cryovials in water bath at 37°C. The ovaries were then immediately perfused with Leibovitz L-15 and 10% FBS for 20 min, thus gradually eliminating the cryoprotectant.

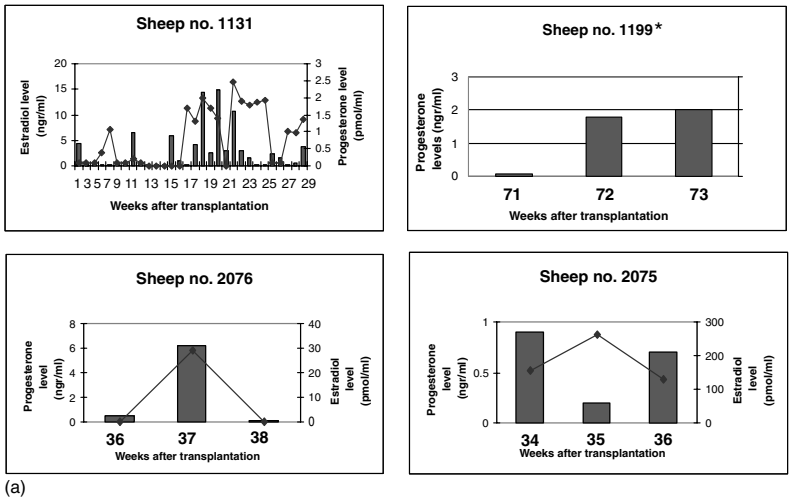
After microvascular transplantation immediate patency was documented in 100% of the grafts. However, after 8–10 days, 77% of the ovaries showed complete occlusion of the anastomosis. While no significant differences were found in the mean values of apoptosis and follicular viability compared to ovarian cortical strip cryopreservation and autotransplantation by TUNEL assay and histology, postoperative FSH levels were much lower in the whole ovary graft group ($P = 0.03$) and similar to preoperative values in animals with patent vessels [44].

After establishing long-term patency of the anastomosis, the next objective was to assess whether the post-transplanted ovaries could respond to in vivo stimulation with FSH and produce viable oocytes. Grazul-Bilska *et al.* treated ewes for approximately 5 months post-transplant with FSH and then the ovaries were removed [49]. In all ovaries, primordial, primary, secondary, antral and pre-ovulatory follicles were found along with fully functional vascularization, which was manifested by the expression of factor VIII, vascular endothelial growth factor (VEGF) and smooth muscle cell actin (SMCA). Proliferating cells were detected in follicles, and the rate of apoptosis was minimal. One ewe had four visible follicles from which three oocytes were collected, but none fertilized. The morphology of autotransplanted and control ovaries was similar. The authors concluded that autotransplantation of intact frozen–thawed ovaries is feasible because vascular and cellular function may be restored.

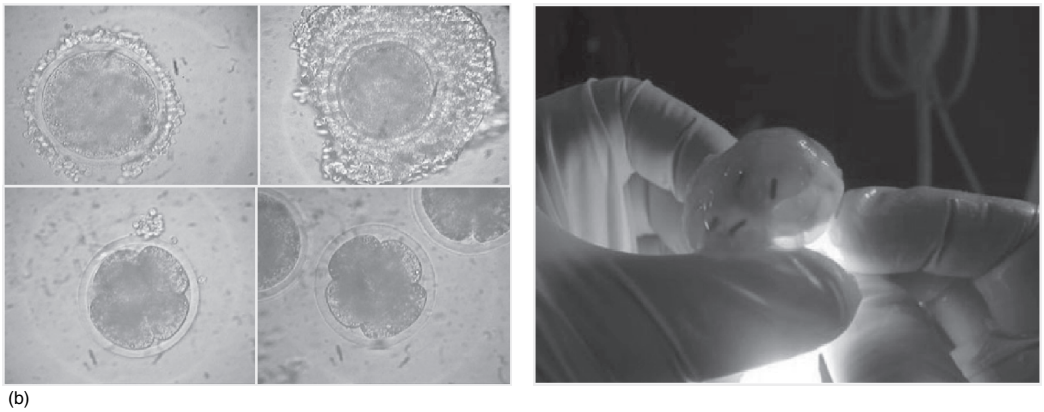
Using both in vitro and in vivo methods, Arav *et al.* showed no significant difference in follicular survival between fresh ovaries and frozen–thawed ovaries, as well as similar histological morphology and normal immunohistochemical expression of factor VIII, suggesting normally restored vascular pedicles [50]. In addition, six oocytes were aspirated from two sheep, and subsequent fertilization and embryo development occurred (Figure 30.1 [50, 51]). Two of the sheep continued to show normal hormonal cyclicity by progesterone levels up to 36 months post-transplantation, and follow-up MRI studies confirmed normal ovarian size and intact vasculature. Furthermore, in a very recent study, Arav *et al.* demonstrated that the follicular and endocrine function lasted up to 6 years post re-transplantation [51]. This is the longest documented functional survival of a frozen/thawed whole ovary. The experiments performed by the Arav group used 8–12-month-old sheep where, after dissection of the right ovarian artery and vein via laparotomy, the ovaries were removed and perfused through the ovarian artery with 4°C University of Wisconsin (UW) solution containing 10% DMSO for 3 min. Freezing was performed using a novel freezing device, the Multi-Thermal-Gradient (MTG; Coredynamics, Ness Ziona, Israel), which utilizes a directional freezing gradient [48, 50]. By advancing the freezing test tube at a constant velocity of 0.01 mm/s through predetermined temperature gradients, freezing was performed at 0.6°C/min until a seeding temperature was reached, and then at 0.3°C/min until –30°C, after which time, the tubes were plunged into liquid nitrogen.

Thawing and grafting was performed 3–14 days later by plunging the Cryovials into a 68°C water bath for 20 s and then into a 37°C water bath for 2 min. The cryoprotectant was removed by reperfusion of the ovarian artery with UW supplemented with 0.5 M sucrose and 10 IU/ml heparin. Ovarian re-transplantation was performed via end-to-end anastomosis of the ovarian artery and vein to the contralateral (the left side) ovarian vascular pedicle of the same sheep via repeat laparotomy. Successful re-anastomosis was documented in five of nine sheep. Progesterone cyclicity was seen 34–71 weeks after transplantation, thus documenting the functional survival of primordial follicles after the freeze/thaw transplant process.

Imhof *et al.* first explored cryopreservation by cannulating the ovarian artery from freshly retrieved porcine ovaries, and flushing with RPMI-1640 solution containing 1.5 M DMSO and 10% human albumin for 30 min on ice [52]. The ovaries were transferred to a programmable freezer at a starting temperature of 4°C, and cooled at 2°C/min to 0°C. The temperature was then lowered by 1.5°C/min to –9°C and then by 0.5°C/min to –40°C. Cooling was then continued at 10°C/min to –150°C. The vials were then plunged into




(a)



(b)

**Figure 30.1** Endocrine function and fertility after cryopreservation and re-transplant of intact sheep ovaries at 2 and 6 years post-transplantation. (a) Hormonal levels 2 years post-transplantation [50]. (b) Follicles by transillumination 6 years post-transplantation [51]. See plate section for color version.

liquid nitrogen. Thawing was performed 3 weeks later by re-warming in air for 2 min before the ovaries were immersed in a water bath at 25°C, and the cryoprotectant was removed from the tissue by washing in saline and fresh medium [52]. Light and electron microscopy were used to evaluate follicular and oocyte survival. Of the primordial follicles, 84.4% in the frozen–thawed ovaries appeared histologically, with 73% of the follicles looking similar to those from the unfrozen contralateral ovary [52].

After success using the porcine model, Imhof *et al.* then cryopreserved and re-transplanted whole ovaries in sheep [53]. Using a similar method for cryopreservation, the authors performed a second laparotomy 3–5 weeks later, and the frozen–thawed ovary was autografted by microvascular end-to-end anastomosis to the contralateral pedicle. Ischemia time before complete re-anastomosis was 30 min. The FSH and progesterone levels were used to evaluate ovarian function. Initially FSH levels kept rising for 3 months but reached normal physiological levels about 6 months after transplantation. Progesterone was first detected 12–14 months after implantation and 2 of 9 sheep resumed normal ovarian function. One sheep achieved a spontaneous pregnancy, with delivery of a healthy lamb [53].

Wallin *et al.* conducted a study to assess methods for the evaluation of viability and function of frozen–thawed whole ovaries [37]. Histology and a viability assays were used to evaluate the ovaries. Fourteen ewes underwent oophorectomy via laparotomy and their ovaries were frozen using the slow cooling

method. A solution containing 1.5 M propanediol, 0.1 M sucrose and 2% human serum albumin in Leibowitz L-15 medium was used as the cryoprotectant in one group of ovaries. Ovaries were stored between 1 week and 9 months. No antral follicles were seen in the cryoprotectant group and edema was noted within the stroma. There was no histological difference between the two groups.

## Vitrification

While many attempts have been made to cryopreserve whole ovaries by slow freezing techniques, there have been few studies that have attempted utilizing vitrification. This method is becoming increasingly recognized as alternative to organ and tissue preservation by slow freezing as it circumvents the mechanical damage cause by ice crystal formation [54].

Fahy and colleagues have described two vitrification solutions that may be useful for whole ovary cryopreservation. The first, VS1, contains 20.5% wt/vol. DMSO, 15.5% wt/vol. acetamide, 10% wt/vol. propylene glycol and 6% wt/vol. polyethylene glycol in a modified Dulbecco's saline (HB1) [55]. The second, VS4, contains 2.75 M DMSO, 2.76 M formamide and 1.97 M propylene glycol diluted in BM1 medium [56]. The two solutions, VS1 and VS4, were compared by Courbiere *et al.* in a sheep model of whole ovary cryopreservation for toxicity to primordial follicles and vessels by collecting ovaries with intact pedicles from 5 to 6-month-old lambs [57]. Each ovary was perfused via the ovarian artery with heparinized Ringer's solution, followed by perfusion and immersion in a bath with VS1 or VS4 solution. Perfusion rate was performed at 0.35 ml/min with a stepwise increase in concentration of cryoprotectant. After perfusion, ovaries were transferred into cryobags containing the cryoprotectant mix and then plunged into liquid nitrogen. After storage, the vitrified samples were rapidly rewarmed in a 37°C water bath, and the cryoprotectant was removed by a reversed concentration gradient perfusion, and then washed in BM1 medium for 5 min [57].

Follicle viability fell from 75.6% ± 1.1% without vitrification to 68.2% ± 1.9% after vitrification with VS1, and from 68.0% ± 3.8% to 60.7% ± 2.4% after vitrification with VS4. While these differences between VS1 and VS4 were not statistically significant, follicle density remained significantly higher in vitrification with VS4 ($P < 0.05$). Histologically, the percentage of normal primordial follicles fell after vitrification, with 25.2% ± 7.0% of follicles remaining normal with VS1 and 53.5% ± 3.2% remaining normal with VS4. There were also more post-vitrification cytoplasmic anomalies with VS4 ($P < 0.05$) but more nuclear and combined anomalies with VS1 ($P < 0.05$). Fractures occurred in vessels during thawing in 3 of 5 cases with VS1 and in 8 of 10 cases with VS4, although the authors noted that the catheters used to re-perfuse the ovarian artery were fitted without difficulty. This study showed that whole sheep ovaries can survive vitrification with good immediate follicular viability via histological evaluation; however, the blood vessels were cryodamaged.

Similar to previous to slow-freezing studies, Courbiere *et al.* performed a follow-up study in 2008, attempting vascular anastomosis to the contralateral pedicle in sheep ovaries either fresh or after vitrification [58]. Successful microsurgical transplantation was performed in both groups, but, not surprisingly, the median ischemia time was significantly longer in the cryopreservation group. Only one out of five ewes undergoing ovarian vitrification recovered endocrine function six months after transplantation compared to four out of five in the fresh transplant group. However, histological evaluation showed total follicle loss in the vitrification group, suggesting that attempts at cryopreservation with vitrification were unsuccessful, despite technical feasibility [58].

## Whole human ovary cryopreservation

Despite the many technical challenges involved in the choice of cryoprotectants and tissue viability after freezing and thawing, there have been several attempts at whole ovary cryopreservation in humans (Table 30.1 [59, 60]). In 2004, Martinez-Madrid *et al.* tested the feasibility of freezing intact human ovaries using a passive cooling device [24]. Ovaries from three premenopausal women undergoing oopohorectomy were resected with their vascular pedicle intact. The ovarian artery was cannulated and the ovary was perfused first with isotonic heparinized solution, and then with a solution of Leibovitz L-15, 10% DMSO and 2% human serum albumin for 5 min at 2.5 ml/min. The ovary was then placed in a Cryovial and cooled at a rate of −1°C/min to −80°C, at which time it was transferred to liquid nitrogen. For thawing, the Cryovial was directly transferred to a water bath at 60°C. To

**Table 30.1** Summary of whole human ovary cryopreservation experiments listed according to type of freezing method, cryoprotectant used and outcomes measured to indicate post-thaw viability

| Study | *N* = | Surgical method | Freezing method | Cryoprotectant | Outcomes measured |
|---|---|---|---|---|---|
| Martinez-Madrid *et al.* [24] | 3 | Laparoscopy | Slow cooling | DMSO | Follicle, stromal cell, vascular viability, histological morphology |
| Bedaiwy *et al.* [25] | 2 | Laparoscopy | Slow cooling (ovaries bisected) | DMSO | Follicle viability, apoptosis |
| Martinez-Madrid *et al.* [31] | 3 | Laparoscopy | Slow cooling | DMSO | Apoptosis, ultrastructural assessment |
| Jadoul *et al.* [46] | 9 | Laparoscopy | Slow cooling | DMSO | Technical feasibility of oophorectomy and freezing |
| Patrizio *et al.* [59, 60] | 11 | Laparoscopy, laparotomy | Slow cooling | EG | Apoptosis, histological morphology |

DMSO, dimethyl sulfoxide; EG, ethylene glycol.

remove the cryoprotectant, the ovary was reperfused at room temperature, with a reversed sucrose concentration gradient of 0.25, 0.1 and 0 M sucrose in L-15 medium.

Viability evaluation by vital fluorescent staining revealed that the percentage of live follicles was 99.4% in fresh tissue, 98.1% after cryoprotectant exposure and 75.1% after thawing. Viability assessment also showed live stromal cells and small vessels after thawing. On histological evaluation, the morphology of follicles and cortical and medullar tissue was similar in all three groups. While this work utilized a much more simplified cryopreservation algorithm than in previous studies, and it did not report on vascular endothelial viability [61], it did suggest that a slow cooling method could be utilized preserving whole human ovaries [24]. Martinez-Madrid *et al*. showed high survival rates of follicles, small blood vessels and stromal cells in an intact human ovary using an accessible cryopreservation protocol [24].

Martinez-Madrid *et al*. have subsequently reported on additional viability assessments in frozen/thawed human ovaries [31]. They assessed apoptosis via TUNEL-assay as well as immunohistochemistry for active caspase-3. No primordial or primary follicles were found to be positive for either TUNEL or active caspase-3, and they found no significant differences in mean TUNEL-positive surface area values between fresh control and frozen/thawed ovaries. Electron microscopy also showed well-preserved ultrastructure, healthy-appearing primordial and primary follicles and normal endothelial cells [31].

In 2007, the same group described the technique of laparoscopic oophorectomy with the intent to cryopreserve the whole ovary and its vascular pedicle [46]. The whole ovary was successfully removed and cryopreserved by arterial catheterization in all nine patients in the study. The authors had two main conclusions: first, that the ischemic interval before cryopreservation must be as short as possible, as this is the time when significant damage is done to the organ; second, that surgeons must leave a long infundibulopelvic ligament (>5 cm) in order to allow easier access to the ovarian vessels during canalization and perfusion of the cryoprotectant.

More recently, Bedaiwy *et al*. described the successful cryopreservation of the human ovary [25]. They performed bilateral oophorectomy in two premenopausal women. In each case, one ovary was cryopreserved intact with its vascular pedicle in the same method as described earlier. Ovaries were thawed 7 days later, and follicular viability and histology were assessed, as well as apoptosis via TUNEL assay and Bcl-2 and p53 protein expression profiles. They found that overall viability of the primordial follicles in the two ovaries was 75 and 78% in frozen/thawed ovaries, and that there were similar primordial follicle counts, absence of features of necrosis and mean values of apoptosis when compared to control ovaries. After demonstrating comparable survival rates and limited molecular alterations, the authors concluded that this represents further evidence that an intact human ovary could be cryopreserved using a slow freezing protocol. However, there has been confusion about whether the

**Figure 30.2** Whole human ovary cryopreservation with the vascular pedicle utilizing the same Multi-Thermal-Gradient device and slow cooling, rapid thawing protocol as described in the text. See plate section for color version.

ovaries in this study were bisected after perfusion with a cryoprotective agent [62].

More recently, we have reported a successful whole human ovary cryopreservation with the vascular pedicle utilizing the same MTG device and slow cooling, rapid thawing protocol described earlier [48, 63] for the sheep (Figure 30.2). With this method, 11 ovaries from premenopausal women undergoing hysterectomy and bilateral salpingo-oophorectomy have been successfully cryopreserved for 48–96 h following either laparotomy, laparoscopic or robotic-assisted laparoscopic oophorectomy [59]. In all cases, the ovarian artery was successfully cannulated for perfusion of cryoprotectant and the contralateral ovary was utilized as a fresh control. A pathologist was unable to distinguish between the frozen/thawed ovary and control in a blinded histological analysis. Immunohistochemistry and Western blot assays showed modest increase in anti-caspase 3 and p53 phospho-serine expression, suggesting a non-significant increase of apoptosis in the frozen thawed specimens [59]. In addition, in three cases, the ipsilateral fallopian tube was also cryopreserved intact with the associated ovary and pedicle. In these cases, the histological architecture was also preserved, suggesting that cryopreservation of the entire adnexa en bloc may be technically feasible [60].

## Conclusions

The survival of reproductive-age women with cancer has dramatically improved over the last several decades and, as a result, researchers and patients are exploring their options for fertility preservation. As cryopreservation protocols have improved and technical challenges related to whole organ cryopreservation have been partially resolved, the prospect of fertility preservation by whole ovary cryopreservation has become more of a reality. While no human studies of whole ovary transplant after cryopreservation have been performed, studies in large animals have been encouraging.

The strategy of whole human ovary cryopreservation has a major potential advantage over the cortical strips: it allows for immediate perfusion of the transplanted organ thereby reducing the ischemic damage, thus theoretically resulting in long-term resumption of ovarian and endocrine function. However, whole ovary cryopreservation may not be a realistic option for many patients due to inherent technical difficulties. The process requires a challenging surgery due to the small diameter of the ovarian artery, further exacerbated by the inadequate length of the vascular pedicle. If the microvascular anastomosis fails, then the whole organ survival is irreversibly compromised, preventing a second attempt at transplantation. This is in contrast to failure of transplanted cortical strips, when another attempt can be performed with the remaining frozen strips.

An issue that remains unresolved is the handling of ovarian tissue containing metastasis from systemic cancers such as leukemia. In the future, patients with malignancies at high risk of ovarian metastasis could have a whole ovary removed and perfused in vitro, to stimulate folliculogenesis in vitro. If successful, oocytes could be harvested for cryopreservation or for fertilization and subsequent embryo cryopreservation.

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Chapter 31

# Ovarian transplantation

## Whole ovary transplantation

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## Introduction

Over the past few decades, considerable attention has been given to the long-term reproductive function of females undergoing cancer treatment. Many treatments, such as non-specific chemotherapeutic agents and ionizing radiation, damage the ovaries and induce premature ovarian failure (POF), making future pregnancy impossible. Indeed, the number of cancer patients at risk of POF who are seeking help to preserve their fertility has increased dramatically.

One method of preserving fertility in female cancer patients is cryopreservation of ovarian cortical strips. In this procedure, strips of ovarian tissue are harvested before cancer treatment, cryopreserved and then thawed for subsequent use. The practice has become well established within the last decade. However, the subsequent use of the frozen–thawed ovarian strips to restore fertility remains a challenge. Transplantation is currently the only available option to restore fertility using cryopreserved ovarian tissue because in-vitro follicular culture technology is far from fully developed in humans, although it has been successful in the rodent model [1].

An option for those undergoing pelvic radiation therapy is fresh whole ovary autotransplantation. Typically, transposition of the ovaries can be performed. However, in cases where the radiation beam involves much of the pelvis and abdomen transposition may not be possible. In this case, the ovary can be surgically removed and immediately implanted into an alternate site that is not exposed to pelvic–abdominal radiation. Currently, this procedure can be done using fresh whole ovaries only. Although whole ovaries have been successfully frozen and later transplanted back into the donors in animal models, the procedures needed to do so safely and successfully in humans are still under development.

In this chapter, we review the state of the art of ovarian transplantation as it pertains to the whole ovary.

## Ovarian tissue transplantation: current status and limitations

In a previous study, we systematically reviewed the literature to assess the outcomes after ovarian tissue transplantation (OTT) in women who were at high risk for POF [2]. In that report, we identified 46 unique cases from 25 studies in which cryopreserved or fresh OTT was performed. In most cases, the transplantation was done using ovarian tissue strips. The indications for the procedure were: treatment for POF ($n = 27$), prevention of POF ($n = 15$), treatment for infertility ($n = 2$) and accidentally ($n = 1$).

The procedure restored spontaneous menstrual cycles for several months in almost all cases. In 23 women with a follicle stimulation hormone level (FSH) of >30 at the time of OTT, ovarian function (OVF) was re-established; the median time to return of function (ROF) was 120 days (range, 60 to –244). Within 6 months of ROF, 4 of the 23 women experienced recurrent ovarian failure. There were insufficient data to evaluate the long-term effects of the transplantations, including OVF (>12 months).

Of particular note, the use of fresh grafts increased the chances for return of OVF and decreased the likelihood for recurrent ovarian failure more so than the use of cryopreserved grafts. Of 25 women who sought pregnancy, 8 had 9 pregnancies 12 months after OTT giving a cumulative pregnancy rate of 37%.

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Live births after autologous transplantation of frozen–thawed cortical tissue have been reported [3–6]. Pregnancies and live births have also been reported after heterologous transplantation of fresh and frozen–thawed ovarian tissue between twins discordant for POF [7–10].

The time needed to re-establish ovarian function after transplantation can take as long as 4–5 months as the follicles usually require at least 120 days to start growing, and another 85 days or so are needed for them to fully mature [2, 11]. The longevity of the grafts after transplantation is variable; OVF may last only for a few months or many years [2]. Although transplantation restores menstrual function, basal FSH levels generally remain elevated after surgery, reflecting poor ovarian reserve [12]. In addition, the oocytes harvested from these transplanted tissues tend to be immature and of poor quality [13]. Of the pregnancies that have been reported after OTT thus far, all occurred within the first year after transplantation, once menstrual function was re-established and FSH levels normalized [3–10]. These observations demonstrate the importance of achieving adequate ovarian endocrine function before attempting pregnancy.

## Post-transplantation ischemic ovarian damage: morphological and functional consequences

Avascular OTT is limited by one important fact – the graft completely depends on the establishment of a new blood supply via the process of neovascularization. Avascular grafts experience an initial period of ischemia after transplantation while waiting for neovascularization to occur and consequently, a large proportion of follicles are lost during this time [14–20]. The main feature in all of the OTT reports that used cortical strips is the limited graft longevity and high risk of recurrent ovarian failure (Box 31.1) [2]. The key reason behind this phenomenon is the fact that most of these experimental transplants were performed without vascular re-anastomosis. As a result, ischemic injury occurred before full revascularization of the transplanted tissue from the surrounding vessels could take place.

The exact mechanism by which the neovascularization process occurs is unknown and so is the time needed for this process to be completed. Theoretically, the shorter the time needed for neovascularization, the longer the graft survival. In animal experiments, the time needed for revascularization was 3 days post-transplantation in mice [21] and up to 1 week in rats [22, 23]. In an in vitro experiment, the neovascularization process was observed after only 3 days following human OTT onto the chorioallantoic membrane of a chick [24]. The ischemic exposure is critical for the survival of not only primordial follicles but also ovarian stroma as well [25, 26].

**Box 31.1** Effects of ischemia on ovarian transplants.

**Morphological**
- Decreased size
- Accelerated fibrosis
- Decreased number of primordial follicle

**Functional**
- Reduced longevity
- Increased basal follicle stimulating hormone (FSH) levels
- Decreased inhibin B level
- Decreased anti-Müllerian hormone (AMH)

It has been estimated that ischemic exposure is associated with accelerated loss of the follicular pool. In a series of xenografting experiments, approximately 25% of the primordial follicles were lost as a result of transplanting cryopreserved xenografts of human ovarian tissue into mice [14]. In autografting experiments, it was noted that ischemic injury was associated with up to a 95% loss of follicular reserve [17, 27]. In addition to follicular depletion, ischemic exposure was associated with abnormal hormonal function of the graft in the form of up to a three- to fourfold increase in FSH levels during the estrus cycle in the sheep animal model. This increase in FSH levels could have occurred if the growing follicles did not produce enough inhibin A [28]. These hormonal changes could also be explained in part by the granulosa cell dysfunction observed in the grafted tissue [29]. In addition, low anti-Müllerian hormone levels reflecting poor ovarian reserve have been reported [30].

## Strategies to prevent post-transplantation ischemic ovarian damage

Many strategies have been devised to minimize the initial post-transplantation ovarian ischemia (Box 31.2).

**Box 31.2** Strategies to nullify the effect of ischemia on ovarian transplants.

**Microvascular anastomosis**

**Neoangiogenic factors**

- Fibroblast growth factor (FGF)
- Transforming growth factor β (TGFβ)
- Vascular endothelial growth factor (VEGF)

**Antioxidant**

- Vitamin C
- Vitamin E
- Erythropoeitin
- Melatonin

**Hormones**

- Follicle stimulating hormone (FSH)
- Luteinizing hormone (LH)
- Human menopausal gonadotropin (HMG)
- Gonadotropin-releasing hormone (GnRH) analogues
- Estrogen–progesterone

**Implantation on granulation tissue**

Steps to improve neoangiogenesis have been successfully applied by experiments that have shown early perfusion of ovarian cortical strips upon their transplantation into granulation tissue [31]. Free oxygen radicals are produced as a result of the ischemia perfusion process. They have the potential to damage cell membranes, endothelial membranes and mitochondrial function [32]. The use of exogenous antioxidants to augment ovarian transplant resistance to oxidative stress-associated damage has been evaluated by many investigators. In one study, for example, ascorbic acid and mannitol reduced surgically-induced ovarian ischemic injury in rats [33]. Moreover, injection of vitamin E before ovarian transplantation improved the follicular survival rate [21]. These preliminary data have not been confirmed by others, however [34]. Sapmaz *et al.* locally administered melatonin and oxytetracycline during intraperitoneal rat ovarian grafting and assessed the effects on graft function. They found that the injection reduced ovarian tissue necrosis [35]. In an in vitro model, Kim *et al.* found that incubating ovarian tissue with ascorbic acid for up to a maximum of 24 h reduced apoptosis [26].

It is expected that neoangiogenic growth factors such as fibroblast growth factor (FGF), transforming growth factor (TGF) and vascular endothelial growth factor (VEGF) somehow aid in the establishment of graft function. Preliminary data from an animal study (monkey model) showed that the most angiogenic growth factor, VEGF, was not associated with improved graft function [36]. This was due in part to the systemic administration of VEGF. Local administration at the transplant site may be more beneficial. In lower animals, it was shown that the invasion of the rat cortex by vessels was associated with a significant increase in the expression of mRNA in the outer cortex for both TGF and VEGF [22].

A wide variety of hormonal treatments designed to be given before or after ovarian grafting have been created in an effort to the consequences of the ischemic exposure. These hormones include, but are not limited to, recombinant FSH, luteinizing hormone (LH), human menopausal gonadotrophins, gonadotropin-releasing hormone (GnRH) agonists, estrogen and progesterone [37]. Data from several animal experiments showed that pre-treating the graft recipient and/or the donor with gonadotrophin stimulation before and after transplantation may have a positive effect on the viable growth follicle rate [21, 38, 39]. However, the impact of such treatments on long-term ovarian function and fertility is still questionable and needs further investigation.

## Whole ovary transplantation

Parenchymatous organ transplantation has been practiced with variable success rates all over the world for many decades, particularly with kidneys and livers. These transplantations are typically heterologous between HLA-matched genetically different individuals. On the other hand, transplantation of reproductive organs can be done via autotransplantation or heterologous transplantation. Autotransplantation is the process of transplanting tissue back into the body such as with OTT or from one part of the body to another in the same individual. With heterologous transplantation, the donor tissue is transplanted into another person. The dynamics and the logistics of whole ovary transplantation are not the same as those for non-reproductive organs.

It has been almost two decades since whole ovary autotransplantation was reported in early human studies. In those reports, ovaries were removed from their pelvic location and immediately transplanted into other sites. The use of heterotopic sites for ovarian

autotransplantation dates back to 1988, when the first case was reported [40]. These authors documented normal follicular growth and regularity of menstrual cycles after the end of pelvic radiotherapy.

Whole ovary transplantation with a vascular anastomosis was proposed as a mechanism to reduce ischemic time and, in theory, prolong the longevity of the graft [41]. In this technique, the whole ovary with its vascular pedicle is removed, cryopreserved, thawed and then transplanted with a microvascular anastomosis into a heterotopic or orthotopic site. Transplantation of an intact ovary with vascular anastomosis reduces the ischemic interval between transplantation and revascularization by allowing immediate revascularization of the transplanted tissue [42].

Bedaiwy *et al.* reported the restoration of ovarian function after autotransplanting intact frozen–thawed sheep ovaries with microvascular anastomosis [43]. Imhof *et al.* autotransplanted whole cryopreserved sheep ovaries with microanastomosis of the ovarian vascular pedicle, which lead to pregnancy and delivery [44]. An intact human ovary with its vascular pedicle could be cryopreserved without affecting the follicular viability, vascular density or molecular integrity of different ovarian components [45].

## Whole fresh ovary transplantation

Fresh whole ovary transplantation with vascular anastomosis has been successfully performed using a wide variety of orthotopic and heterotopic recipient sites. In addition, a number of vessels have been used in a wide variety of animal models. These include pelvic vessels such as the ovarian artery and iliac artery, parietal vessels such as the inferior epigastric vessels and extrapelvic vessels such as the carotid vessels [39, 46]. In our preliminary experience with Merino sheep, the revascularization process was compromised in approximately 50% of the cases when fresh ovaries were transplanted [41].

All human whole ovary transplantation has been performed using fresh ovaries. A limited number of human studies [9, 47–49] have attempted this exhausting and technically challenging approach (Table 31.1). A team of surgeons is needed, and the tissues must undergo intraoperative microscopic evaluation. This procedure is usually attempted in cancer patients who need to start chemo- or radiotherapy without delay, but other patient groups as monozygotic twins discordant for POF [9] or Turner's syndrome [49] have benefited from it.

The upper arm was the first recipient transplantation site tested in humans. There are two reports of successful whole fresh ovary transplantation in the upper arm that was done prior to sterilizing pelvic irradiation [47, 48]. Leporrier *et al.* created a cavity in the forearm arm for the ovary using a testicular prosthesis that was inserted 3 months before the transplantation. In that report, the ovary remained function for the next 16 years [47]. In the second report, the transplantation process was performed in the context of radical hysterectomy for early stage cervical carcinoma. The ovary remained functional for at least 1 year after transplantation [48].The procedure was unfortunately followed by local vault malignant recurrence. No long-term follow-up has been reported. Mhatre *et al.* successfully implemented the technique in two patients with Turner's syndrome (ovarian function was restored and the patients developed secondary sexual characteristics) [49]. An abdominal pfannenstiel incision was used in both cases.

More recently, Silber *et al.* culminated their efforts in ovarian transplantation in monozygotic twins discordant for POF by reporting the first full-term pregnancy obtained using orthotopic whole fresh ovary transplantation with microvascular anastomosis [9]. A fresh ovary from the fertile twin was implanted in her monozygotic twin with POF.

## Whole cryopreserved–thawed ovary transplantation

Whole frozen ovary transplantation with microvascular anastomosis was first described in rats by Wang *et al.* [39]. They described successful vascular transplantation of frozen–thawed rat ovaries, which were transplanted along with the reproductive tract, in 4 of 7 (57%) transplants; these transplants survived for $\geq$60 days, were ovulatory and resulted in 1 pregnancy. Ovarian function was restored in 100% of cases when fresh organs were transplanted [50]. Following their success, Courbiere *et al.* [51] described cryopreservation (vitrification) of whole ovaries with vascular pedicles in 5 to 6-month-old sheep. There was no statistically significant difference in follicle viability or normal primordial follicle rates between the ovaries exposed to two cryoprotectants (VS1 and VS4 containing dimethyl sulfoxide, formamide, and propylene glycol) and those

**Table 31.1** Studies reporting whole fresh ovary transplantation in humans

| Reference | No. of patients | Ovarian volume and site | Indication for transplantation of ovarian tissue | Outcomes |
|---|---|---|---|---|
| Leporrier, 1987 [47] | 1 | Whole ovary, heterotopic | Hodgkin's disease | Ovarian cycles remained regular despite radiotherapy, and follicle growth occurred normally |
| Hilders *et al.*, 2004 [48] | 1 | Whole ovary | Cervical cancer | • Normal blood flow in the anastomosed artery and vein. Cyclic swellings of the upper arm without major discomfort<br>• Documented follicular activity at different stages<br>• Adequate blood flow and follicular activity of the transplanted ovary |
| Mhatre *et al.*, 2005 [49] | 2 | Whole ovary, orthotopic: Case 1, vascular pedicle; Case 2, avascular transplantation | Turner's syndrome | • Case 1: serum estradiol showed a significant rise from the pre-transplant value of <20 pg/ml to >50 pg/ml<br>• Case 2 showed after 2.5 years: developed uterus with endometrium, normally functioning transplanted ovary and her native streak gonads. At the conclusion of 2.5 years, the patient is having spontaneous menstruation, ovulation and excellent growth of secondary sexual characteristics |
| Silber *et al.*, 2008 [9] | A pair of monozygotic twins discordant for premature ovarian failure | Whole fresh ovary with microvascular anastomosis | Premature ovarian failure | First successful spontaneous pregnancy |

that were not. The same observations were maintained before and after vitrification with the cryoprotectant solutions.

Although reports describing autotransplantation of frozen–thawed sheep ovarian cortex resulting in a pregnancy [52] and prolonged normal hormone production date back to the 1990s [18], it was not until 2003 when the first report of successful cryopreservation and transplantation of an intact ovary in sheep (defined as return of hormonal functions) occurred [43]. Successful pregnancy and delivery of a lamb in sheep was reported by Imhof *et al.* in 2006 following autotransplantation of whole cryopreserved ovaries with microanastomosis of the ovarian vascular pedicle [44].

We have used deep inferior epigastric vessels, while others used the ovarian vascular pedicle, to vascularize frozen–thawed ovaries with success in sheep [43, 53]. The same success was also reported in rabbits [54]. In sheep, the success was suboptimal due to venous thrombosis or a torn artery [41, 44, 55]. We have found evidence of endothelial cell damage caused by the freeze–thaw process or by the ischemic time until successful re-anastomosis [43].

The challenge of whole ovary cryopreservation and transplantation technology is not only the surgical technique but the cryopreservation protocol for an entire organ. Such a protocol should ensure that the cryoprotectant(s) evenly diffuses throughout the entire ovary. In addition, the frozen ovary should survive the thawing process and maintain functionality after transplantation. Imhof reported that 18 months after transplantation the follicular survival rate was <8% [44]. Other authors reported an even lower follicular survival rate (6%) and the depletion of the entire follicular population after whole ovary cryopreservation and transplantation [56]. Although ovarian vessel thrombosis is a potential complication, its incidence is higher when vitrified ovarian tissue is used.

Similarly, in a more recent study in ewes, it was shown that immediate vascular patency was achieved in all ewes and maintained in seven of eight

cryopreserved and three of four control grafts. Functional corpora lutea were identified in 3 ewes (1 control; 2 cryopreserved) 18–25 weeks after grafting. In addition, inhibin A levels indicated resumption of follicular development in four cryopreserved and one control ewes; however, castrate gonadotrophin levels persisted in five cryopreserved and two control ewes. The main prominent feature of this whole ovary transplantation experiment is the fact that primordial follicle density was significantly reduced following grafting in both cryopreserved and non-frozen ovaries [57].

Although transplantation of whole cryopreserved–thawed ovary was not performed in humans, cryopreservation of a whole ovary using a slow-freezing protocol has been successfully attempted [45]. The results showed both vascular and follicular integrity upon thawing after freezing and thawing. More recently, a multi-gradient-freezing device was used with promising results [58]. In that study a high follicular viability, normal histological architecture and preserved vessel integrity were reported, supporting the potential for vascular re-anastomosis.

## Technical aspects of whole ovary transplantation: harvesting approaches

The procedures used to harvest ovaries for subsequent cryopreservation or transplantation should be modified to reflect the need for a healthy and adequate pedicle. Consequently, surgeons must ensure that the pedicle is long enough so that the ovarian artery and veins can be skeletonized and sutured to recipient vessels of a similar diameter. During this process, ischemia time must be minimized [42]. Although open surgery can help diminish ischemia time, a minimally invasive laparoscopic approach is the preferred approach when the ovary will be frozen and subsequently transplanted [59]. These approaches can facilitate the dissection of the ovarian pedicle up to the pelvic brim, a technique that has been used for a long time in ovarian transposition [19]. Using laparoscopy in cancer patients ensures better wound healing, which is consistent with the patients' need to start their chemotherapeutic cycles immediately [42].

We have summarized the technical and microsurgical principles of the laparoscopic approach for ovariectomy [59]. These included severing the utero-ovarian ligament before advancing cephalad through the mesosalpinx and ending at the infundibulopelvic ligament, which should be dealt with last to minimize ischemia time. All through the procedure, sharp dissection and suturing are preferred to electrocoagulation, which may induce desiccation of ovarian tissues through thermal injury to the vascular walls. The length and width of the vessels can be maximized by ligating the ovarian vessels as proximal to the origin as possible. An endobag is placed through the 10 mm trocar to deliver the ovary with its vascular pedicle outside the peritoneal cavity.

An extended port incision, which helps avoid crushing the ovary and the blood vessels against the narrow port site, can be used. Another approach, though technically challenging, is the transvaginal route. We reported one case of human ovarian harvesting using this approach, where the researchers combined oophorectomy with vaginal hysterectomy [59]. In order to minimize the ischemic time, we suggest handling one ovary at a time for cryopreservation and saving the infundibulopelvic ligament and the ovarian vessels until the very end of the procedure. We found that the dissection of a long portion of the ovarian vessels via the vaginal route may not be as easy with laparoscopy. The ovary and the uterus on one side has to be released in order to obtain a longer portion of the pedicle. With the advent of laproendoscopic single site surgery (LESS), it is expected that the harvesting of ovaries for fertility preservation will be even less invasive.

## Whole ovary freezing protocols

Several challenges need to be overcome before an ideal intact ovary cryopreservation approach can be developed. Currently, we lack cryoprotective agents that can adequately perfuse into the relatively large tissue masses and prevent vascular injury following intravascular ice formation [42]. The freeze–thaw protocols used today still require further optimization. Some cryoprotective agents lead to zona hardening and meiotic spindle depolymerisation, which may lead to aneuploidy [60].

Modifications of slow-freezing cryopreservation protocols were implemented in ovarian tissue freezing as an integrated strategy for fertility preservation. These modifications mainly entailed increasing the concentration of sucrose used as non-penetrating cryoprotectant and replacing sodium with choline. These modifications improved survival rates by 80% [61].

Cryoprotectants must adequately penetrate the stroma and granulosa cells without causing cryoprotectant toxicity. Newton *et al.* demonstrated the importance of the diffusion rate and the diffusion temperature [14, 62]. Ice crystal formation must also be minimized by choosing optimal freezing and thawing rates. The cryoprotectant that will maximize permeation capacity and minimize toxicity and ice crystal formation is specific to each cell and tissue type [63]. An effective technique of cryoperfusion where the cryoprotectant is perfused into the ovary via the ovarian artery was proposed [64–66]. This technique lead to acceptable follicle survival rates and relative ovarian function restoration with one case of restored fertility [43, 44, 51, 55, 65]. However, the loss of tissue viability amongst these studies was common.

Thus, in the ovary, it is a matter of balance between the stroma, the follicular cells and the oocytes [67]. The standard method for human ovarian cryopreservation is slow-programmed freezing using human serum albumin-containing medium and propanediol, dimethyl sulfoxide or ethylene glycol as a cryoprotectant, with or without sucrose [67].

Martinez-Madrid *et al.* described a cryopreservation protocol for intact human ovary with its vascular pedicle [68]. Ovarian perfusion with cryoprotective solution and slow freezing with a cryofreezing container was performed. Rapid thawing of the ovaries was carried out by perfusion and bathing with a decreased sucrose gradient. High survival rates of the follicles (75.1%), small vessels and stroma as well as a normal histological structure were documented in all the ovarian components after thawing [68]. No post-thawing induction of apoptosis was observed in any of the cell types, assessed by both the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) and immunohistochemistry for active caspase-3 [69, 70]. Transmission electron microscopy confirmed that the majority (96.7%) of primordial follicles were intact and that their endothelial cells had a completely normal ultrastructure after cryopreservation [71]. The percentage of active caspase-3-positive endothelial cells was <1%. It appears that in large mammals and humans, cryopreserving a large, intact ovary may prove more problematic than cryopreserving a small ovary from a small animal due to the difficulty of achieving adequate cryoprotective diffusion and vascular injury caused by intravascular ice formation.

## Transplantation techniques for whole ovaries with microvascular anastomosis

Several transplantation techniques of intact ovaries have been described. Jadoul *et al.* suggested that by applying clips on the utero-ovarian ligament, the ischemic interval could be reduced prior to ovarian artery perfusion with heparinized solution and cryoprotectants [42]. They also recommended that a microsurgeon and biologist should attend the procedure and have a cooled sterile table with a stereomicroscope and microsurgical instruments ready. They would enable surgeons to proceed immediately to ovarian pedicle dissection and ovarian artery catheterization, thereby reducing ischemic time. Also, severing the infundibulopelvic ligament at the end of the procedure could also help minimize ischemic time [59].

This approach has several limitations, especially in regards to its ability to cryopreserve an entire organ. In order to restore fertility after cryopreservation of ovaries and testes in small laboratory animals, vascular transplants require technically challenging microsurgery to deal with the small blood vessels [72–74]. In ewes, using an aortic patch, the ovarian artery was re-anastomosed to the carotid artery during autotransplantation of an intact ovary with its vascular pedicle [75]. A primate model was tested for an orthotopic transplantation technique using a microsurgical re-anastomosis of the ovarian blood vessels [76]. Both techniques have a rather limited chance for application in clinical practice since an aortic patch is used for the anastomosis [41].

An intact human ovarian autotransplantation was reported in a pair of 38-year-old monozygotic twins discordant for POF [9]. The donor ovary was removed laparoscopically from the fertile sister by dividing the infundibulopelvic ligament at its base to maximize the length. Using minilaparotomy, the donor's ovarian veins (3.0 mm in diameter) were anastomosed to the recipient's ovarian veins with 9–0 nylon sutures, and the donor's ovarian arteries (0.5 mm in diameter) were anastomosed to the recipient's ovarian arteries with 10–0 nylon interrupted sutures. A normal-appearing blood flow through the ovarian vessels of the transplanted ovary was observed after an ischemic period of 100 min. Subsequently, the recipient twin had 11 regular menstrual cycles. At day 427 after transplantation, she became pregnant and gave birth to a normal healthy baby girl. This case demonstrated the feasibility of using whole ovary transplantation between

monozygotic twins who are discordant for POF to restore fertility in the affected twin [9]. Should intact human ovary cryopreservation be optimized, the same approach could be adopted for autotransplantation of intact cryopreserved–thawed ovary with a vascular pedicle (Table 31.1).

Despite this success, determining the suitable recipient vessel for this technique awaits experimental proof. In 2007, guided by the results of animal experiments, we provided a model for human intact ovary autotransplantation based on the human vascular anatomy [59]. The neck, pectoral region, antecubital fossa, lower part of the anterior abdominal wall and the inguinal region are possible transplantation recipient sites in humans. The carotid vessels, the cutaneous branches of the internal mammary vessels, the antecubital vessels, the inferior epigastric vessels and the femoral vessels are anastomosed to the ovarian vessels in their respective regions. Based on safety, amenability to monitoring, caliber, accessibility, liability to trauma, surgical anatomy and cosmetic factor, the deep inferior epigastric vessels stand as the best available heterotopic option.

A discrepancy in diameter between the ovarian vessels and the recipient vessels is an important challenge to the subsequent patency of the anastomosis. The sudden change of caliber between the cut end diameters of the vessels, encountered in approximately one third of anastomoses, may cause turbulence in the blood flow, thus predisposing the vessel to platelet aggregation [77]. The choice of the technique of re-anastomosis is affected by the degree of discrepancy. There is no ideal technique to deal with every size discrepancy, and choices may have to be individualized according to the specific case and body area [78]. Options range from dilatation with the use of a jeweller's forceps [77], in the case of a simple discrepancy of $<$1.0 : 1.5, to using the oblique cut, fish-mouth cut or end-to-side anastomosis when discrepancies exceed 1.0 : 1.5 [77]. Sleeve anastomosis is performed when discrepancies are larger and when the upstream donor vessel is smaller than the recipient vessel [79].

Given the straight course and wider caliber of the ovarian vessels, anastomosis of the ovarian vessels in humans is expected to be less technically challenging, provided that the recipient vessels are similar in diameter [59]. Based on animal studies, we suggested the use of three different microvascular anastomosis techniques that base the anastomosis on the caliber of the ovarian and the deep inferior epigastric vessels: end-to-end, end-to-side and fish-mouth anastomosis [59]. The anastomosis of the ovarian vessels to the deep inferior epigastric vessels was performed using 8–10 interrupted sutures (9–0 or 10–0 prolene). Obviously, end-to-end anastomosis appears to be the ideal approach to performing the anastomosis procedure, with a patency rate $>$60% [59]. However, if vascular discrepancy between the ovarian vessels and the inferior epigastric vessels is inevitable, end-to-side anastomosis should be used. Vascular clips [42], sutureless approaches, glues and adhesives and laser-assisted anastomosis are potentially useful [78].

## Microvascular thrombosis after transplantation

In the only reported intact fresh human ovary transplantation, the vascular anastomosis was functional for more than a year after transplantation [9]. In the animal experiment of Bedaiwy *et al.* , long-term patency was lost in 8 of 11 transplanted sheep ovaries due to thrombotic events in the re-anastomosed vascular pedicle [43]. Imhof *et al.* reported that in 6 of 8 ovaries, the major ovarian vessels were free of thrombosis, with the structural integrity of the ovarian stroma largely retained 18–19 months after transplantation [44]. These complications motivated Jadoul *et al.* to highlight the necessity of cryopreserving the contralateral ovarian cortical tissue when whole ovary cryopreservation is attempted, until the results of ovarian transplantation are validated [42].

Another alternative was discussed by Yin *et al.*, who suggested that one of a pair of ovaries should be left *in situ* so that an intact pedicle is available for exchanging the sterilized organ with the frozen and thawed ones, once the patient is ready for autotransplantation [50]. It also allows for the possibility that the ovary left behind may not be totally damaged by the chemotherapy. There are many reports of pregnancies after chemotherapy.

## Heterologous whole ovary transplantation

A case of an allograft between two non-identical twins was recently reported by Donnez *et al.* [80]. They used ovarian cortical tissue from the donor sister that had already been the donor of bone marrow for a transplant. Although this was done with cortical tissue, these concepts can apply to whole ovary transplantation. The recipient had received chemotherapy, total

body radiation and bone marrow transplantation. The recipient developed spontaneous cycles after receiving the transplant. Furthermore, two oocytes and two embryos were obtained. Since the recipient of the ovarian tissue had also been the recipient of bone marrow, the patients were able to avoid long-term immunosuppressive medication. The possibility of acute graft rejection and long-term immunosuppressive complications in the mother, such as infection and obstetrical complications, may limit its use in allogeneic transplantation in patients that have not had a bone marrow transplantation and potential donor. The introduction of an ovary into a patient with a different genetic makeup may make this procedure unacceptable to societies that do not accept donor oocytes.

## Conclusion

The current evidence suggests that whole frozen–thawed ovary transplantation may be successful in humans in the future. However, the significant depletion of the primordial follicle reserve observed after transplantation in animals is a major problem that needs to be further addressed at the experimental level prior to attempting that approach in humans. With the recent success of whole ovary transplantation with microvascular anastomosis, reproductive organ transplantation researchers may need to learn from other transplantation settings. Safer cooling techniques and new operative steps that minimize vascular thrombosis are needed. Multi-team approaches should be expanded [42]. Future research should operate within the framework of the patient's needs, namely fertility and sexuality.

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Chapter 32

# Molecular and cellular integrity of cultured follicles

David F. Albertini, Gokhan Akkoyunlu and S. Samuel Kim

## Introduction

With the advent of culture models for studying the process of ovarian folliculogenesis some 40 years ago, opportunities arose for the more systematic evaluation of the factors that regulated ovarian function [1]. The initial focus of studies using cultured follicles emphasized two of the then widely recognized roles of the follicle in mammals: the production of ovarian steroid hormones and of viable oocytes during the process of ovulation. As our understanding of the molecular and cellular complexity of this tissue compartment has evolved and deepened, so too has the need to redefine the major functions of the follicle at both local ovarian and systemic levels in the context of reproduction in mammalian species, especially as it relates to the origins and treatments for human infertility [2]. Thus, a shift in the motivation to use cultured follicles in humans has taken place owing primarily to the rapidly evolving field of fertility preservation. Through an interesting turn of events dictated by the need to maintain and propagate human oocytes that would be capable of supporting term gestations, a dire need has been recognized that would enable optimization of follicle functions under in vitro conditions in order to realize ovarian capacity for young women who have had their fecundity seriously compromised as a result of genetic, environmental or iatrogenic life-sparing treatments such as those involved with the management of cancer [3–5].

The introduction of technology that permits the cryopreservation on ovarian tissues has opened the prospect of sustaining and storing primordial follicles from individuals that could at a later time be thawed and subjected to prolonged culture. Exactly what conditions will be required to sustain follicular function to support the growth phase of oogenesis has yet to be worked out, as are the factors that would normally be involved in this protracted and oocentric phase of folliculogenesis. Working out conditions that could recapitulate the structural, molecular and cellular properties of the follicle in vitro remains a major challenge in the area of fertility preservation, as does the development and implementation of novel technologies that would permit a reproducible and reliable assessment of oocyte integrity. It is the intent of this chapter to review methods that have been in practice for the evaluation of follicle culture integrity and to point out the strengths and deficiencies of these methods. Finally, the techniques that loom on the horizon which could meet the criteria necessary for monitoring follicular integrity will be considered, as new sentinels or biomarkers could predict the developmental capacity of oocytes for the field of fertility preservation.

While direct measures of oocyte developmental competence remain a lofty goal, more recent efforts to characterize this aspect of follicular integrity have tended to rely upon indirect ways to avoid unnecessary damage to the oocyte that might already have been compromised by ex vivo conditions and/or cryopreservation [3, 6, 7]. For this reason, we propose a viewpoint of follicle integrity that emphasizes the syncitial heterocellular character, which includes the somatic granulosa and theca elements and offers multiple parameters for assessment that in the larger picture may better represent the overall quality of the enclosed oocyte [8]. This viewpoint is further supported by the recent appreciation of the level of signaling and metabolic integration that reflects the

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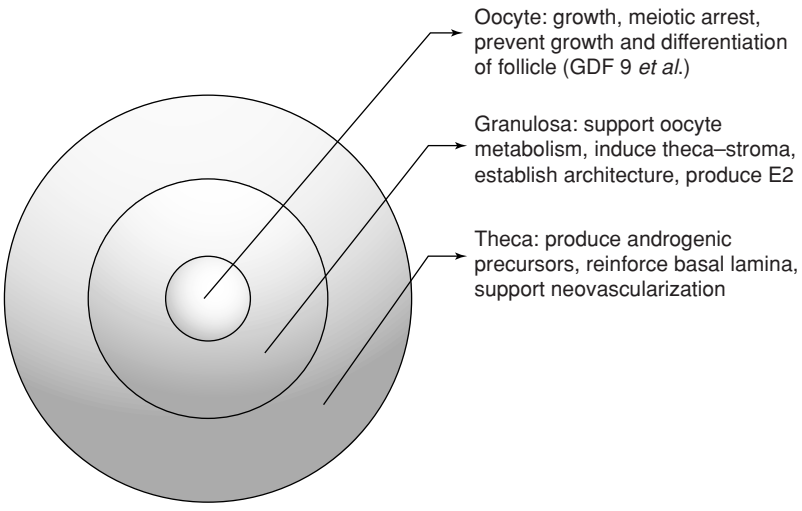


**Figure 32.1** Diagram illustrating the gradient of influences from the oocyte to granulosa and thecal compartments of the ovarian follicle that serve as guideposts for monitoring the cellular and molecular integrity of follicles maintained in culture. Note that the primary function of oocyte secreted factors such as growth differentiation factor-9 (GDF-9) is to prevent the proliferation (hyperplasia) and differentiation (hypertrophy) of the steroidogenic functions of the follicle prior to and following ovulation. Thus, signs of steroidogenesis in culture are likely to reflect a loss in this command function of the oocyte resulting in impaired viability and developmental competence.

continuity of both negative and positive feedback mechanisms between the oocyte, granulosa and theca cells that comprise the ovarian follicle [2]. While this perspective excludes formal contributions from the surrounding stromal compartment within which the follicle develops, the role of the stroma cannot be overlooked with respect to the contributions that it makes during the course of in vivo folliculogenesis in relation to thecal lineages including the microvasculature of antral stage follicles. Thus, what follows is a summary of the current and future biomarkers that may be useful in evaluating the integrity of cultured follicles. We begin with somatic components of the follicle and finish with those assayable properties of cultured oocytes that must be analyzed before the use of oocytes in assisted reproductive technology (ART) applications such as in vitro fertilization (IVF), embryo culture and transfer.

## Evaluating somatic cell components of the follicle

The traditional array of biomarkers for the ovarian follicle has emphasized endocrine performance, especially as related to the biosynthesis and secretion of estradiol [9, 10]. Built on the classical two-cell model, assays that monitor the production of thecal androgens in response to luteinizing hormone (LH) and granulosa cell-derived estradiol in response to follicle stimulating hormone (FSH) remain the mainstay for evaluating follicular integrity under in vivo and in vitro contexts [8]. It can be argued, however, that these valid and predictive biomarkers for the endocrine health of the follicle overshadow the most relevant attribute that directly bears on the overall developmental status and health of the oocyte (Figure 32.1).

Thus, the major phases of oogenesis, during which both oocyte growth and preparations for the completion of meiosis and fertilization occur, occupy the earliest stages of follicle development between the activation of the primordial stage and entry into the secondary or antral stage when estrogen production commences upon the induction of aromatase activity in response to FSH [11–14]. That the latter property appears commensurate with the rapid expansion of the theca and granulosa by a burst of cell proliferation is often identified as a measure of follicular health, but the essential question is how do these FSH-induced attributes of the follicle, increased proliferation and aromatase activity, influence the terminal stages of oogenesis if the goal of culturing follicles is to obtain high quality oocytes?

There can be no question that the measure of any organ or tissue culture system is the health and well-being of its constituent cells. Moreover, as in many other developmental systems that are highly regulated in a stage-specific fashion, the ovarian follicle engages the basic cellular properties of differentiation, proliferation, survival and death, and each of these apply to both the theca and granulosa at select stages of follicular development (Table 32.1).

It is not surprising then that the most commonly used measure of cultured follicle integrity is hypertrophy over time, whether follicles are cultured under

**Table 32.1** Summary of major functional properties for ovarian follicles that have been used in the assessment of tissue integrity[a]

| Property | Follicle stage | Biomarker | Vital |
|---|---|---|---|
| Quiescence | Primordial | Chromatin/PTEN | Yes (Hoechst 33342) |
| Proliferation | Primary, secondary | BrdU, PH3, MPM2 | Yes (Click-IT) |
| Apoptosis | Secondary, antral | TUNEL, caspase 3 | Yes (Hoechst 33342) |
| Autophagy | Primordial (? others) | Beclin/ATG | Yes (LysoTracker Red, acridine orange) |
| Differentiation | Antral | Aromatase, LHr | No |

[a] Note that many of the biomarkers employed to date draw on the use of immunocytochemical and histological assays requiring tissue destruction. Also, not all markers are pertinent to all stages of folliculogenesis. Some examples of vital biomarkers that are under development for determining cultured follicle integrity are shown in the last column.

adherent two-dimensional conditions or within matrices of various kinds that retain a three-dimensional architecture [15–20]. While standard protocols deploying assays for cell proliferation (3H-thymidine, BrdU incorporation or cell cycle markers such as phosphohistone-3, PCNA, etc.) or apoptosis (TUNEL, caspase-3) offer postscripts for the relative fraction of viable cells within a follicle (Table 32.1), in the end these are crude and retrospective assays that add little to the immediate needs of the clinician requiring a more real-time assessment of follicle integrity. Towards this end, several new probes have gained usage in the evaluation of tissue culture models that take advantage of the speed, sensitivity and spectral properties of microplate readers. This new generation of reagents permits resolution of metabolic activity, including reactive oxygen generation, cell proliferation and even identification of rapid-versus-slowly dividing cells within an organ or tissue culture using multi-well formats, which should avail the optimization of conditions that support oogenesis. Moreover, as discussed below, the link between DNA damage sensing and repair is fast becoming a major determinant in the assessment of follicle integrity as it relates to both somatic and germ cell components, and these assays have introduced a range of sensitivity and precision that will materially advance the field of follicle culture.

As with most in vitro systems, culture environments create adverse conditions that are known to affect DNA integrity, often due to the generation of free radicals in response to high oxygen tension [21]. Given these deleterious side effects of culture environments, genomic integrity is one area of follicle evaluation that has received little attention and requires closer inspection. Many new reagents are available for evaluating the cascade of events associated with the

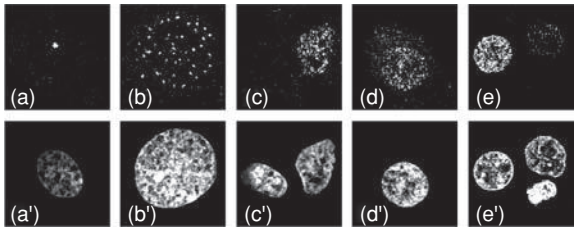


**Figure 32.2** Range of DNA double-strand breaks detected in bovine granulosa cell cultures observed before or after exposure to cyclophosphamide. Top row (a–e) illustrates staining profiles after labeling with gamma-H2AX antibody that reveals solitary foci (a), multiple foci (b) and various patterns (c–e) that presumably reflect earlier stages in DNA lesioning prior to foci formation as indicative of active sites of DNA repair. Bottom row (a′–e′) demonstrates total chromatin. Scale bar represents 10 μm.

detection of DNA damage, as well as the activation and completion of the DNA-repair pathway that should be active in both somatic and germ cells of the follicle. We have recently been exploring the components of this pathway in the mammalian ovary and find that a variety of insults – ranging from advancing maternal age, reactive oxygen species (ROS) generation during culture and exposure to chemotherapy agents such as cyclophosphamide – bring about rapid and reversible changes in the degree of DNA damage and repair in ovarian cells. As shown in Figure 32.2, granulosa cells isolated from bovine ovarian follicles provide a useful culture system for evaluating DNA damage by immunofluorescence microscopy.

A wide variety of reagents are now available that detect epitopes that appear in response to spontaneous or induced double-strand breaks. Amongst these, antibodies that recognize histone modifications that occur at the site of strand breaks reveal both the extent and magnitude of lesions in the form of foci of varying number and size within single cell nuclei. By fixing

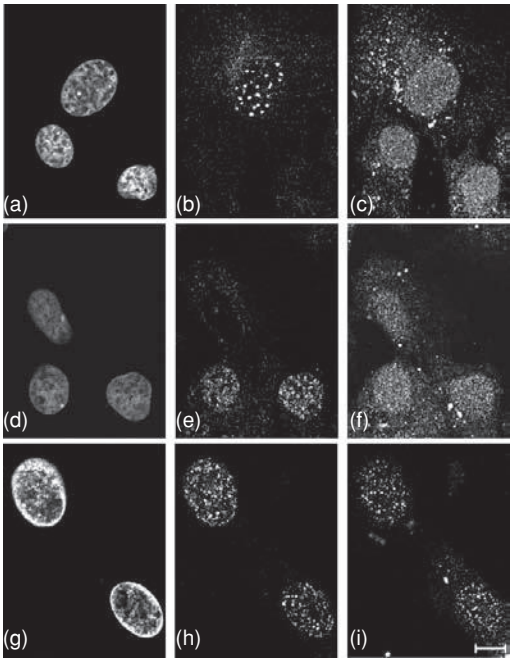


**Figure 32.3** Bovine granulosa cells cultured in the absence (a–c) or presence (d–i) of cyclophosphamide for 4 h (d–f) or 24 h (g–i) that have been fixed and stained for the demonstration of total chromatin (a, d, g), gamma-H2AX (b, e, h) and RAD51 (c, f, i). With progressive repair, as evidenced by the formation of discrete gamma-H2AX positive nuclear foci, cytoplasmic RAD 51 assumes an intranuclear location coincident with the sites of DNA double-strand breaks. Scale bar represents 10 μm.

cells in response at various times following exposure to the chemotherapy agent cyclophosphamide, these foci appear in increasing numbers that can be quantified as the mean density/nucleus. This gives us an understanding of the relative time course of the induction of DNA damage and the rate of repair upon drug removal or after altering culture conditions (Figure 32.3).

When used in combination with reagents that detect components of the repair complex, such as RAD51 or BRCA1, further insight into the DNA repair capacity can be obtained. This approach is applicable to sections of ovarian tissue or cultured follicles, and thus adds an important dimension to the assessment of DNA integrity in both somatic and germ line components of the follicle under in vivo or in vitro conditions.

## Center stage: supporting and maintaining oogenesis

In the context of fertility preservation, the singular objective of follicle culture is to provide an ex vivo context within which the growth and maturative stages of oogenesis can proceed unabated and without inflicting damage to the oocyte as it acquires the capacity to support embryonic development [18, 22, 23]. As alluded to above, to a certain extent a paradox emerges, since the most valid predictors of the success of follicle culture will reside in the ability to document that indeed oocyte quality has been established and maintained for whatever duration of follicle culture is needed to achieve this objective [5, 24–26]. Thus, while a substantial body of evidence supports the idea that folliculogenesis consumes upwards of 100 days in humans [1, 2, 8], we remain ignorant as to the exact chronology and duration of events that are required to support the entire process of oogenesis as it occurs within the confines of the follicle. Moreover, it remains unclear as to whether the process of folliculogenesis reflects a developmental continuum or one that is punctuated by episodes that attend to key steps in oogenesis dictated intrinsically or from cyclic variations in gonadotropin availability or expression of receptors for LH or FSH [27–30]. Answers to these questions will inform future approaches in this field in conjunction with the emergence of assays that more directly target and report upon the quality of the oocyte within cultured follicles.

As noted above, short of assessing the ability of oocytes to complete meiosis, engage successfully in fertilization in vitro and support pre-implantation development [1, 7, 31], there are few biomarkers that could safely be considered as reliable or efficient in determining oocyte quality.

Those that have been proposed – such as metabolic substrates, visual indicators that rely on the use of fluorescent reporters or other vital indicating dyes ( Table 32.1) – pose limitations pertaining to assay sensitivity, free radical generation and metabolic perturbations respectively, thus precluding their general utility. For these reasons, non-invasive and non-perturbing optical assays remain a cornerstone for the assessment of oocyte quality in materials that will be subjected to IVF and embryo culture as a measure of developmental competence.

The more promising indirect biomarkers are those that monitor the integrity of the interaction between oocytes and granulosa cells, whereby both the differentiative state of the oocyte and its surrounding granulosa cells can be assayed [31, 32].

Based on the tenet that establishing contact between the oocyte and granulosa is realized through

the anchoring of transzonal projections at early stages of follicluogenesis, the effectiveness of these connections is likely to be a direct reflection of the metabolic synergy required to acquire meiotic competence and preserve the oocyte in a developmentally competent state for the duration of culture [27, 33]. Earlier studies using mouse ovarian follicles emphasized the importance of minimizing FSH exposure as this resulted in the precocious resumption of meiosis, a consequence that would materially exacerbate the problem of oocyte aging [17]. Thus, more recent efforts to achieve maintenance of oocyte health during oocyte culture have viewed the inclusion of FSH at inappropriate times to be hazardous to the oocyte while advancing follicle differentiation to the next level [1, 14, 18, 30]. Interestingly, these adverse effects of FSH appear to be due to a direct action on the transzonal projections (TZPs) as they are rapidly and irreversibly retracted and may delimit the availability of nutrients and/or signals that operate during both the acquisition of meiotic competence and maintenance of meiotic arrest [34]. It may well be that, besides identifying more suitable media conditions favoring oocyte viability, other factors will be discovered that effect a more stable interaction between oocytes and granulosa cells based upon the composition of the extracellular matrix [6, 10] and whether or not cultures are maintained in a two- or three-dimensional context [18–20, 35]. One final factor that is likely to contribute to the stability of TZPs is growth differentiation factor-9 (GDF-9). Originally discovered as a critical oocyte produced factor required for the development of secondary follicles [36], GDF-9 was shown to be essential for the establishment of TZPs in mouse knockout models [37]. Whether exogenous recombinant forms of GDF-9 or related oocyte signaling molecules will facilitate metabolic synergy remains to be demonstrated, but this variation in culture conditions would be worth exploring in seeking alternative strategies that would preserve the native architecture of the follicle in a fashion consonant with support of oocyte development and metabolism [38].

Maintaining cellular interactions within the follicle is relevant to the effects of ovarian tissue cryopreservation or primordial follicle organization. The hyperosmotic stress induced by cryoprotectants in both slow freeze and vitrification protocols may disrupt cell interactions in distinct ways. As shown in Figure 32.4, slow freeze protocols tend to perturb the stromal–basement membrane interface whereas vitrification impacts the interface between the oocyte and surrounding granulosa cells.

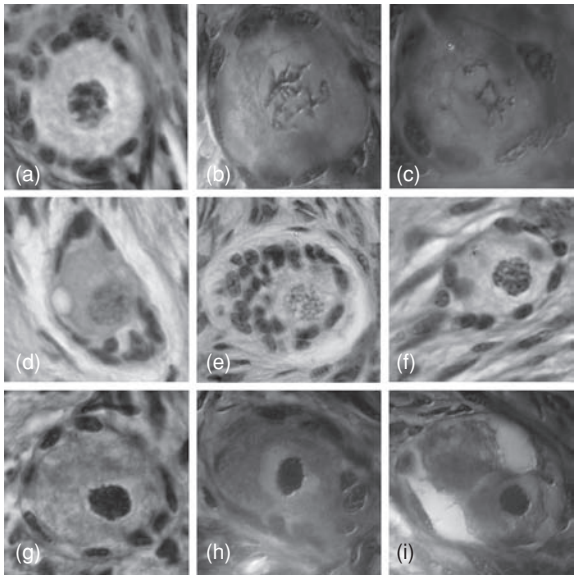


**Figure 32.4** Micrographs illustrating chromatin integrity in routine histological preparations of primordial follicles from fresh (a–c), slow frozen (d–f) and vitrified (g–i) bovine ovarian cortical strips. The top row (a–c) demonstrates the appearance of diffuse diplotene germinal vesicle chromatins within primordial follicles viewed under conventional bright field (a) or Nomarski differential optics (b, c); note the distended configuration of chromatin towards the nuclear margin and cytoplasmic homogeneity. Samples thawed for 30 min following slow freeze retain a fibrillar character that fills the nucleus (d–f), whereas those recovered following vitrification consistently demonstrate condensed chromatin retracted from the nuclear periphery (g, h) and retraction of the oocyte cortex from the interface with granulosa cells (i).

Importantly, these preliminary observations on bovine cortical strips also suggest that the linkage of chromatin to the nuclear envelope may be at greater risk after vitrification when compared to tissues recovering from slow freeze. Together, measures of intercellular and intracellular protein interactions will represent an important direction for further studies on follicle integrity.

## Future prospects for assessing the integrity of cultured follicles

The evaluation of follicle integrity for future applications in fertility preservation will require changes in our conceptual and technological approaches to this problem. Throughout the course of this chapter, the emphasis dictated by the need to obtain healthy oocytes of high developmental competence warrants

conceptualizing folliculogenesis as an oocentric process rather than one that fulfills the endocrine functions so vital to reproductive physiology [8, 9]. In this sense, future research into the phases of follicular development that are focused on supporting the growth stage of oogenesis, as well as in anticipation of the resumption and completion of meiosis, are likely to uncover complex feedback pathways that are subject to regulation by the patterns of gene and protein expression resident within the oocyte [39]. Defining what these factors are, and how their presence signals transformations in the behaviors of granulosa and thecal cells, should reveal arrays of biomarkers that in the end reflect the ongoing role of the oocyte in determining its ultimate fate [2, 40–44]. This may require application of more sensitive methods for the detection of metabolites and substrates whose concentration is maintained in a microenvironment more reminiscent of what is present within the intact ovary. This goal is achievable if the potential of microfluidic technologies is realized for cultured follicles as it is being realized for other model developmental systems requiring protracted periods of time in vitro.

A second dimension of future considerations for the ex vivo maintenance of ovarian follicles concerns that of time [3, 4, 18]. While the exact course of events and their chronology that govern the development of oocytes is not known, it is likely to require some weeks for the entire process to be brought to fruition. Besides being of an invasive nature, rote biochemical and molecular studies – while providing large quantities of data that might be expected to serve some value as "biomarkers" – are unlikely to contribute in a major way to our understanding of the epigenetics of oogenesis. This rapidly evolving area of contemporary biology has already entered the front-lines of human ARTs [18, 20, 24] given the well-documented effects that various in vitro procedures have on the software that drives the cytoplasmic and nuclear genomes of the early embryo, all of it inherited from the process of oogenesis. Our biological clocks are changing the way in which epigenetic modifications of the oocyte genome play out during the natural course of folliculogenesis [2, 27], warranting use of caution in future applications of follicle culture technology that would be relied upon to preserve oocytes for individuals whose germ line may have been compromised as a result of natural aging or the adverse effects of cancer treatment [4, 18].

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Chapter 33

# In vitro growth systems for human oocytes

## From primordial to maturation

Evelyn E. Telfer and Marie McLaughlin

## Introduction

Tissue banking of ovarian material is being increasingly offered to a variety of patients as a means of fertility preservation [1]. This tissue comprises of thin cortical surface biopsies that contain predominantly immature primordial follicles and currently is the only option to restore fertility using this tissue is by transplantation [2]; however, this may not be a viable option for all patients [3]. Increased options to maximize the potential of this tissue to restore fertility could be realized by the development of in vitro systems to support complete growth from the early primordial stages through to maturity. This technology would have many therapeutic applications, including the production of competent oocytes for assisted reproductive technology (ART); a model system to determine toxicological effects on germ cell development; a method for the assessment of cryopreserved ovarian tissue prior to transplantation; as well as providing an experimental model to address basic scientific questions concerning human oocyte development [4, 5]. Complete oocyte development in vitro from the primordial stage has been achieved in mice, but the larger size and longer growth period of human follicles has made the inter-species translation of these techniques difficult. Recently progress has been made in defining conditions that support different stages of human follicle development in vitro [6, 7], and these advances bring the prospect of achieving a complete in vitro system that supports oocytes from primordial to maturation closer [8]. This chapter deals with our current understanding of in vitro development of human oocytes and highlights the gaps that need to be bridged to achieve a complete in vitro growth (IVG) system.

## Follicular development

Female reproductive function requires cyclical development and maturation of ovarian follicles on a background of continuous activation from the pool of primordial follicles (Figure 33.1a). Primordial follicles are formed prenatally and represent a finite population of germ cells from which recruitment for growth will take place throughout the woman's reproductive life. Follicular growth and development involves a series of complex and precisely regulated events. It is characterized by transition stages that begin with: (1) initiation of primordial follicle growth and development to the preantral follicle stage; (2) the formation of antral follicles where expansion to the pre-ovulatory or Graafian follicle is associated with granulosa cell proliferation and antral fluid accumulation within the basement membrane; and (3) rupture of the Graafian follicle releasing a cumulus–oocyte complex at ovulation in response to the mid-cycle luteinizing hormone (LH) surge (Figure 33.1a).

During its growth within the follicle the oocyte is held in meiotic arrest, but as it grows it must acquire the ability to resume meiosis (meiotic competence) and the ability to support fertilization and embryonic development (developmental competence). Thus, the oocyte is dependent upon the local environment within the follicle for subsequent function as a gamete. The development of follicles is regulated by a complex mixture of inhibitory and stimulatory endocrine, paracrine and autocrine signaling by the somatic cells (granulosa and surrounding theca cells) enhanced by a range of oocyte specific regulatory factors mediated through bi-directional communication within the follicle [9].

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Published by Cambridge University Press. 

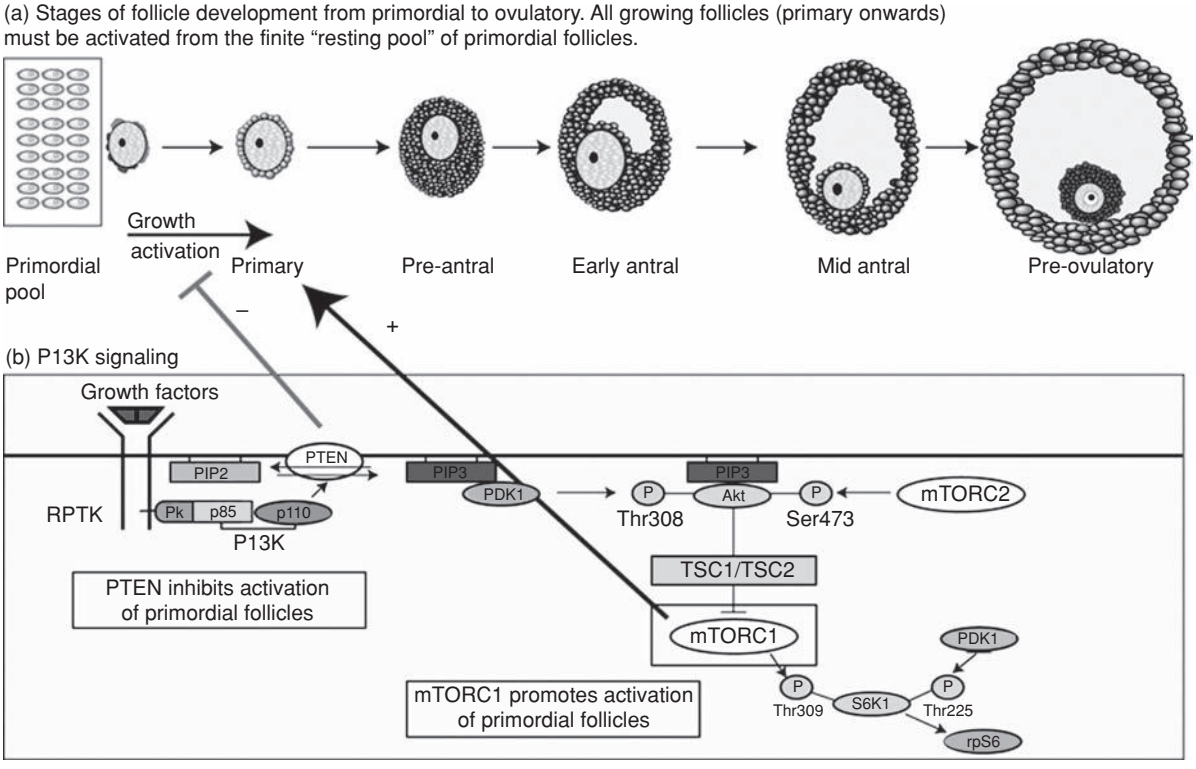


**Figure 33.1** (a) Digrammatic representation of follicle growth from the non-proliferating pool of primordial follicles. Primordial follicles are continuously activated into the growing population where they become primary follicles consisting of an oocye arrested at the dictyate stage of prophasel of meiosis (yellow) surrounded by granulosa cells (green). Primary follicles undergo oocyte growth and granulosa cell proliferation and differentiation (purple) when they form an antral cavity. Antral follicles continue to grow and granulosa cells differentiate into two subpopulations of cells: (1) cumulus surrounding the oocyte (blue); and (2) mural lining the wall of the follicle (orange). Exact timings for this developmental sequence to occur in humans are not known but estimations suggest several months. However, it is not known whether the growth profile is continuous or whether there are "resting" phases through follicle development. (b) Simplified version of the PI3K pathway. The factors initiating this process are largely unknown but a body of evidence is emerging to show that the phosphatidylinositol-3'-kinase (PI3K-AKT) signaling pathway is a major regulator of early follicle/oocyte development and that components of this pathway are involved in controlling the rate of activation from the non-growing population of follicles. The phosphatase PTEN converts PIP3 to PIP2, which negatively regulates PI3K activity. Signaling mediated by PI3Ks converge at PDK1. PDK1 phosphorylates Akt and activates it. Akt can phosphorylate and inactivate tuberous sclerosis complex 2 (TSC2 or tuberin), which leads to the activation of mTOR complex (mTORC1). mTORC1 can phosphorylate (activate) S6K1. S6K1 subsequently phosphorylates and activates rpS6, which enhances protein translation that is needed for cell growth. mTORC1 can be inhibited pharmacologically with Rapamycin and stimulated by leucine. The manipulation of this pathway could have important clinical applications in the field of fertility preservation. See plate section for color version.

In humans the female germ cell population is formed before birth and it is accepted that it is not substantially replenished during adult life [10]. At any age the majority of follicles within the ovary will be at the primordial stage of development, and this represents a pool which is continually depleted during reproductive life [11, 12]. The relative abundance and lack of differentiation of primordial follicles makes this population an ideal choice for IVG to obtain fertilizable oocytes for potential use in ART and fertility preservation programs [5, 13]. The ability to develop these immature follicles fully in vitro would have several basic and clinical applications integral to fertility preservation.

The capacity of immature mammalian oocytes to develop fully in vitro has been demonstrated in rodents with the birth of pups from in-vitro matured oocytes derived from murine cumulus-oocyte complexes [14], primordial follicles [15, 16] and cultured primary follicles [17]. However, this has yet to be successfully repeated in humans or domestic species where follicles undergo a protracted developmental period in vivo. A great deal of basic scientific progress has been made in developing systems designed to

support the partial growth of human follicles and several developmental milestones have been achieved, namely, follicle activation [6, 18–22] pre-antral follicle growth [6, 7, 19, 21, 23–25], follicle differentiation [6, 7] and oocyte maturation [26, 27]. These advances have been made using both fresh and cryopreserved human tissue. In the clinical setting progress has been made with the ability to apply in vitro maturation (IVM) techniques to immature human oocytes with subsequent in vitro fertilization (IVF) of these oocytes leading to pregnancy and live births [28–30]. Despite the successes, stumbling blocks still exist in putting all of these systems together to achieve complete IVG. A major difficulty associated with prolonged culture is oocyte degeneration associated with the disruption of the contact between the oocyte and its companion somatic cells. Therefore it is important to understand how culture conditions impact on the maintenance of appropriate cell interactions during follicle/oocyte development.

## Activation and growth of immature follicles

Central to our understanding of female fertility, reproductive aging and developing IVG systems is knowing how the "resting" population of primordial follicles is regulated. While some local factors regulating the initiation of primordial follicle development have been identified, such as anti-Müllerian hormone [31], the underlying mechanisms involved are largely unknown [32]. A growing body of evidence is now emerging to show that the phosphatidylinositol-3'-kinase (PI3K-AKT) signaling pathway is a major regulator of early follicle/oocyte development and that components of this pathway are involved in controlling the rate of activation from the non-growing population of follicles (Figure 33.1b) [33]. A recent study showed that the phosphatase PTEN acts within the oocyte as a negative regulator of PI3K-AKT and suppresses initiation of follicle development in mice, with global activation of primordial follicles demonstrated in mice with an oocyte-specific Pten knockout [34]. This work also highlighted that PI3K-PDK1 (3-phosphoinositide-dependent protein kinase-1)-Akt-S6K1 (p70 S6 kinase 1)-rpS6 (ribosomal protein S6) cascade in oocytes controls ovarian aging by regulating survival of primordial follicles [34, 35]. Activation of S6K1-rpS6 is largely dependent on the mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that regulates cell growth and proliferation in response to growth factors and nutrients [36]. Recent studies have implicated mTORC1 signaling in regulating dormancy and activation of the primordial follicle population, which is equally important as PTEN/PI3K signaling [35, 37]. Thus there is now an accumulation of evidence to show that PTEN and mTORC1 play important roles both in initiating and regulating progression of follicle development, with PTEN actively suppressing and mTORC1 promoting activation (Figure 33.1b). Manipulation of these pathways could lead to improvements in IVG systems by supporting increased activation in a coordinated manner. Current work in our laboratory is focusing on these processes [38, 39].

In humans quiescent primordial follicles are continuously activated to grow and this is independent of physiological status [40, 41]. Activation of growth is marked by the gradual transformation of the flattened epithelial cells surrounding the oocyte into cuboidal cells which proliferate forming a multilaminar structure in which the germ cell will mature (Figure 33.1a) [4, 11, 42]. In the human ovary, initiation of growth and early follicle development is believed to be an extremely protracted process taking many weeks to complete [2, 11, 28]. However, whether this lengthy period of time accurately represents the definitive rate of somatic and germ cell development or whether in vivo growth is modulated by ovarian molecular constraints is unclear. What is certain is that sustained normal follicle development critically depends upon intercellular communication between the growing oocyte and the developing granulosa [43]. This communication takes the form of developmentally regulated cytoplasmic projections which extend from the membrane of the granulosa and traverse the zona pellucida terminating close to the oolemma frequently forming gap junctions on the oocyte surface [44, 45]. In vivo follicle development comprises of a series of events which occur sequentially and take varying lengths of time to complete. During the protracted transformation of a primordial follicle into a primary follicle the oocyte does not increase in size [11, 46]. However, at the point of antral cavity formation, significant oocyte growth has been achieved [4, 9]. To achieve competency, the fully grown oocyte must undergo nuclear maturation and cytoplasmic differentiation, processes which occur chiefly in concert with granulosa cell proliferation during pre-antral follicle growth [4, 9, 47]. The ability of an oocyte to complete

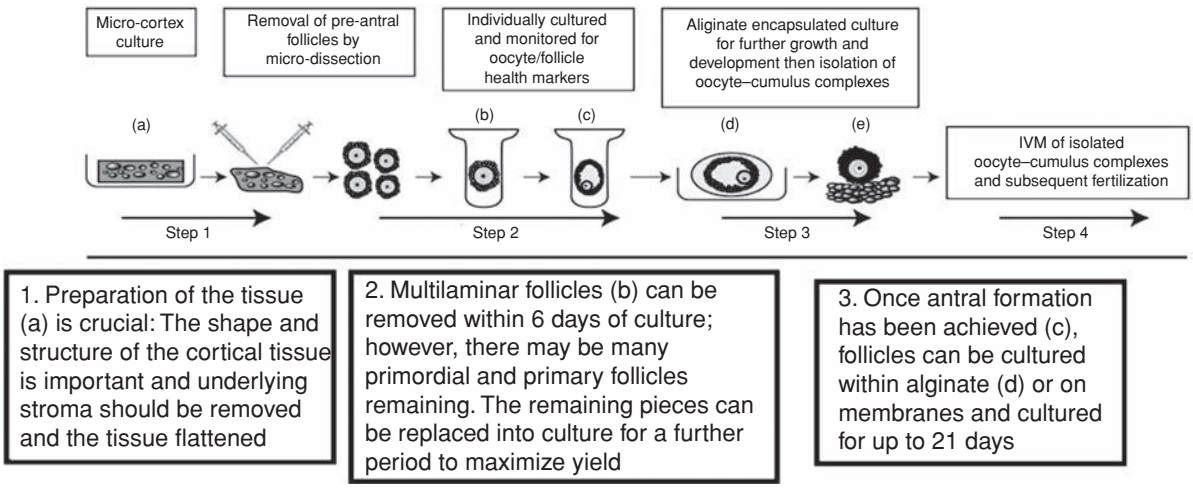


**Figure 33.2** Proposed multi-step culture system for activation of human primordial follicles and subsequent follicle/oocyte development. The stages required for a multi-step culture system are as follows. Activation of primordial follicles within cortical strips (a). Removal of all growing follicles and most of the underlying stromal cells increases the rate of activation [6]. Flattened strips are cultured free floating in medium containing human serum albumin (HSA), ascorbic acid and basal levels of follicle stimulating hormone (FSH) [6]. Once follicles have reached multilaminar stages they are isolated mechanically using needles and cultured individually. Isolated follicle culture is to support development from pre-antral to antral stages (b). The addition of activin at this stage results in improved follicle development and increased antral formation (c) [6]. Follicles of similar stages that have been grown in vivo have been isolated and grown with alginate drops [7] (d), and oocytes grow to almost full size within a total of 30 days [7]. The final stages of oocyte growth and development could be achieved by culturing the oocyte and its surrounding somatic cells outwith the constraints of the large follicle (e). See plate section for color version.

meiosis is acquired after its ability to resume the process; moreover, oocyte size is a determinant in reaching metaphase II [48, 49]. Growing oocytes accumulate cytoplasmic organelles and these are dispersed to the periphery of the cell in readiness for fertilization and pre-implantation embryo development; only oocytes that have completed both cytoplasmic and nuclear maturation are capable of fertilization and subsequent embryo development [50]. The aim of in-vitro follicle development or IVG is to achieve developmentally competent oocytes. Therefore culture systems must provide an environment that will sustain oocyte growth and support cytoplasmic and nuclear maturation but without the necessity to develop large follicular structures [13]. Attempts to recapitulate in vivo development (timings and size) in vitro have not been successful and therefore strategies to improve IVG systems should concentrate on optimizing oocyte growth within the context of surrounding somatic cells in the shortest time possible.

## Strategies to support follicle development in vitro

Various approaches have been taken to promote early human follicle development in vitro using fresh [6, 18] and thawed–cryopreserved [18, 20, 51] human cortical tissue. It is clear that to achieve complete in vitro development of human oocytes a multi-step culture system needs to be developed. The follicle functions both as an endocrine structure and as a vehicle to support oocyte growth and development. In optimizing such a culture system, the focus should be on oocyte development which may not require the development of large follicular structures but rather the maintenance of appropriately differentiated somatic cells in contact with the developing oocyte. The multi-step approach has been designed to support the changing requirements of the developing oocyte and its surrounding somatic (granulosa) cells and the steps involve: (1) culturing small pieces of ovarian cortex to support activation of primordial follicles [6, 18, 21]; (2) isolation and culture of growing pre-antral follicles to achieve oocyte growth and development [6, 7, 19, 23, 25]; (3) aspiration and maturation of oocyte cumulus complexes [26, 52, 53]. Our work has been the first to combine these steps and advances have been made in the first two steps [6]. Figure 33.2 illustrates our proposed multi-step IVG system to produce competent human oocytes from ovarian cortical tissue. The current status of each of these stages will be discussed in the following sections.

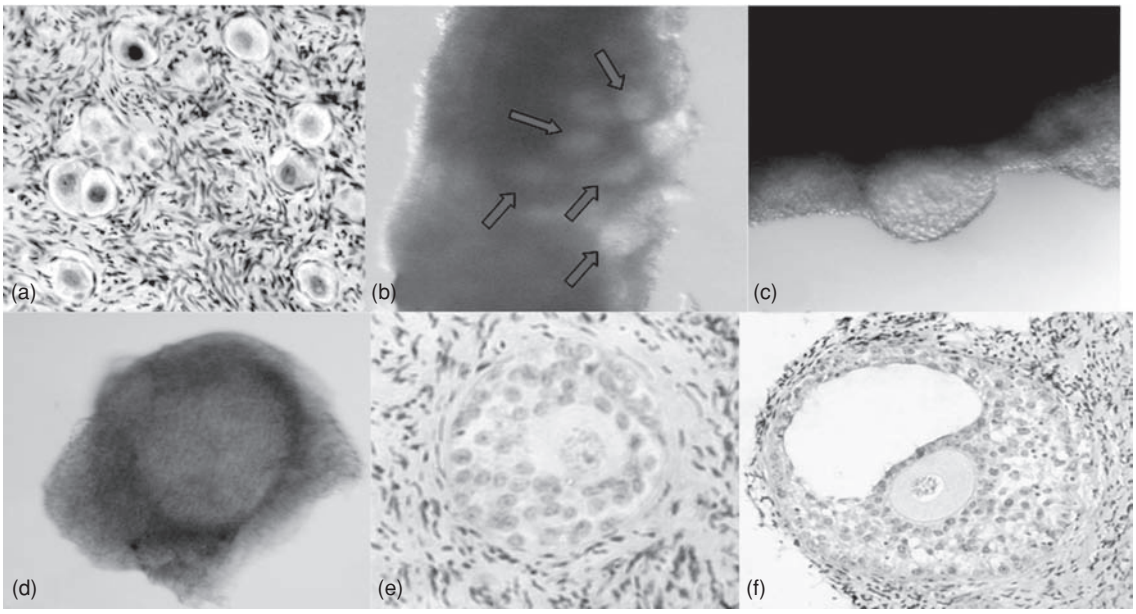


**Figure 33.3** (a) A cluster of quiescent follicles in freshly fixed human ovary. (b) After 6 days in vitro, growing follicles (↑) appear on the surface of a cultured fragment of human ovarian cortex. (c) A growing follicle protruding from the edge of a fragment of cultured human cortex. (d) Intact secondary human mechanically dissected with presumptive theca layers attached. (e) Histological image of a secondary human follicle fixed after 6 days in vitro growth within a cortical fragment. (f) Histological image of human antral follicle fixed after a total of 10 days in vitro growth. See plate section for color version.

## Culture of ovarian cortical biopsies

In fertility preservation programs many young women (particularly pre-pubertal girls) opt for cryopreservation of ovarian cortical biopsies. The majority of viable follicles within this tissue are at the quiescent primordial stage (Figure 33.3a). Therefore the priority for developing an IVG system must be to optimize activation of primordial follicles in vitro and support early follicle development. Recent work from our laboratory [6] has shown that human primordial follicles grow well within mechanically loosened cortical pieces and can develop to multilaminar pre-antral (secondary) stages within 6 days. The culture conditions in this system differ from those described in earlier studies [18, 19], as the culture medium is serum free and no supporting matrix is present. Preparation of the tissue is crucial to success and this involves removal of most of the underlying stromal tissue so that the cultured pieces consist of predominantly ovarian cortex containing primordial and primary follicles (Figures 33.2 and 33.3a). When these small pieces of human ovarian cortex are cultured there is a significant shift of follicles from the quiescent to the growing pool over short culture periods of 6–10 days [6, 38, 51], an observation repeated in cattle where extensive primordial activation has been reported within 2 days in vitro [54–56] indicating that activation results from a release of intraovarian factors that act to inhibit the initiation of follicle growth [37, 55]. Observations made during IVG and confirmed by histological analysis in both human and bovine models demonstrate that the growth of follicles in tissue fragments is differentiated, with the rate of growth enhanced in follicles lying adjacent to tissue edges (Figure 33.3b,c) compared to follicles embedded within dense stroma [McLaughlin and Telfer, unpublished observations]. Moreover, when excessive stroma is removed from tissue pieces prior to culture, the rate of follicle growth increases (Figure 33.3 b,c) [6, 38, 51].

Cortical strip culture removes follicles from the in vivo endocrine and paracrine processes regulating growth rate. However, follicles will still be subject to the effect of follicle interactions and the influence of stromal cell factors. It is clear that tissue shape and stromal density are important factors that regulate follicle growth initiation in vitro, as solid cubes of cortical tissue show a lower rate of growth initiation [18]. In contrast, when stromal cells are removed and the

tissue is cultured as flattened "sheets," the initiation rate is greater and follicles grow more quickly [6]. The physical environment of the follicles within the cortical tissue affects their response to stimulatory and inhibitory factors and therefore influences their ability to grow [57].

## Culture medium to support activation and early growth

The optimum culture medium that supports activation and early growth has yet to be developed. The first culture systems used fetal calf serum (FCS) or human serum-containing medium but this has now been replaced by defined media substituted with human serum albumin (HSA) and a combination of insulin, selenium and transferrin (ITS) with the addition of ascorbic acid to minimize cell death [58–60]. The choice of basal culture medium includes MEM alpha, Waymouths medium and McCoys 5A, with our preference being for McCoys 5A [6]. The most basic medium appears to support activation and growth, and the addition of growth factors such as activin at this stage leads to less activation [61], whilst in bovine systems increased insulin leads to greater activation [55]. Further information is needed on how specific factors affect activation and stromal cell support but a promising approach, which may lead to improvements in the number and quality of growing follicles available for harvesting, is directly manipulating key signaling pathways that control activation (Figure 33.1b). This is now a major focus in our laboratory [38, 39].

Once follicle growth has been initiated within the strip, follicles can develop to multilaminar stages. Growing follicles do not survive well within the cortical environment and it appears to be inhibitory to further growth resulting in a loss of follicle integrity and oocyte survival [6, 19]. Therefore, in order to develop further, follicles require to be released from the cortical stromal environment and cultured individually [6, 38, 51].

## Isolation of growing human follicles

Isolation of pre-antral follicles from cultured cortical tissue can be achieved by mechanical dissection, enzymatic isolation or a combination of both. Enzymatic isolation commonly uses collagenase and DNase to liberate primordial and pre-antral follicles from stromal tissue and yields many more follicles than mechanical dissection [62–65]. However, collagenase can exert a species-specific effect which has been associated with follicle damage and poor survival in large mammals [67, 68], but this may be overcome by modified techniques and new purified enzyme preparations including Liberase [69–71]. Mechanical isolation using fine needles has the advantage of preserving follicular integrity by maintaining the basal lamina and thecal layers of the follicle (Figure 33.3d). However, the yield is low and the procedure protracted and laborious due to the dense fibrous cortical tissue in the ovaries of large mammals, particularly in the human where follicles are embedded in the tough fibrous cortex and, as such, are relatively inaccessible. Enzymatic [19, 24, 25], partial enzymatic [7, 71] and mechanical [6, 23] isolation methods have been used to dissociate human pre-antral follicles from the cortical stroma with the resulting follicles being cultured for up to 4 weeks.

The progression of human follicles following isolation from the cortex is remarkable. In the presence of FSH, enzymatically isolated secondary human follicles can differentiate, become steroidogenically active and complete oocyte growth in 30 days [7]. Furthermore, quiescent follicles activated to grow within cultured fragments of cortex and mechanically isolated as secondary follicles (Figure 33.3e) become steroidogenic and undergo differentiation after a 10 day in vitro period with and without activin (Figure 33.3f) [6]. These observations would appear to confirm that local ovarian factors indeed inhibit development of follicles: the question remains as to whether the growth rate in vitro is accelerated or if it is growth without the brakes on. Further studies on growth rates are required and it is essential to determine how growth rate affects oocyte quality.

## Culture of isolated human follicles

Culture systems designed to support the development of isolated human follicles can be divided into several categories with some degree of overlap. The defining aspects of current culture systems are: (1) the developmental stage of follicles on harvesting; (2) the type of material support employed; and (3) the length of the in vitro period. Although it is possible to enzymatically retrieve viable human primordial follicles from cortical tissue, prolonged culture and survival of individual follicles has been unsuccessful with gross degeneration observed [24]. Whether this is due to enzymatic

damage of the basal lamina or the necessity of these follicles to be in groups with stromal support or a combination of factors is unclear; indeed, it has been noted that the presence of stromal cells can improve the growth of cultured human primordial follicles indicating that support of extrafollicular cells is vital during initial growth [5, 6, 72]. A recent study of ovine primordial follicle culture reports progress after using lectin and kit ligand (KL) to promote oocyte granulosa cell aggregation and oocyte development [73], an encouraging step towards defining the conditions required to support the unilaminar to multilaminar transition in cultured follicles of large mammals.

Growing follicles isolated for in vitro development can be derived from several sources; thawed–cryopreserved tissue, cultured cortical fragments or uncultured ovarian tissue. In vitro growth of thawed–cryopreserved tissue will be discussed later. Although the technique of harvesting in-vitro activated follicles at the secondary stage is still evolving and much remains to be optimized, there are several advantages to selecting these follicles for further development in vitro, including homogeneity and abundance of population, relative lack of apoptotic sequelae [74, 75] and reduced likelihood of disease infiltration [3]. Using our two-step culture technique unilaminar human follicles are capable of growth, differentiation and steroidogenesis after a total in vitro period of 10 days and normal oocyte morphology is maintained [6, 38, 51]. There are very few studies reporting the development of secondary follicles isolated from fresh non-cultured human cortical tissue. However, progress is encouraging with growth, differentiation, steroidogenesis and complete oocyte growth being achieved within a total of 30 days [7]. Optimal results require the inclusion of a basal concentration of follicle stimulating hormone (FSH) (1.0–1.5 IU/ml), and successful culture is promoted by material support of the follicular unit while in vitro to prevent migration of the somatic cells away from the oocyte.

Measures to support the three-dimensional follicular architecture and thereby maintain intrafollicular cell association in vitro have been used in the culture of mammalian follicles for almost two decades and it has been demonstrated, largely using rodent systems, that the use of suspension cultures, mineral oil sheaths, hydrophobic membranes and follicle encapsulation result in the promotion of follicle growth and the attaining of developmental milestones [5]. When culturing large mammalian follicles the use of "v"-shaped micro-well plates has allowed the maintenance of three-dimensional follicular architecture in vitro while promoting growth and differentiation in bovine [76–78] and human follicles [6, 38, 52] with antral formation occurring within 10 days. Follicle differentiation has also been reported in bovine follicles embedded in collagen gels and cultured for 13 days [79] and, using a combination of media thickened with polyvinylpyrrolidone, a macromolecular supplement and microporous membranes, one live calf was produced from immature bovine follicles cultured for 14 days [80]. In addition to "v"-shaped micro-well culture plates, follicle encapsulation in alginate hydrogels has been used to support secondary human follicle growth in vitro [7]. The novelty of alginate encapsulation is that it is believed to mimic the extracellular matrix in vivo in terms of its ability to facilitate molecular exchange between the follicle and the culture medium, while its flexibility can accommodate cell proliferation but its rigidity prevents dissociation of the follicular unit. It would appear that the rigidity of the alginate capsule is of vital importance to follicle development as inhibition of growth and reduced steroidogenesis have been reported in murine follicles embedded in 1.0 and 1.5% alginate gels, respectively [81, 82], whereas fully grown human oocytes have been produced using 0.5% gels [7]. The ability of the reported systems to promote human follicle growth in vitro from the earliest stages at rates that are accelerated in comparison to the in vivo environment is indeed promising. The next step is to ascertain whether the oocytes produced in these systems are capable of IVM and to determine whether the altered growth is deleterious to oocyte epigenetic health and normality.

## Culture of thawed cryopreserved human follicles

If IVG systems are to have a clinical application within fertility preservation they must be capable of supporting development from cryopreserved tissue. The viability of human follicles isolated from cryopreserved tissue was established over 10 years ago [83], and in the past decade human ovarian cryopreservation and tissue re-implantation has resulted in live births [84, 85]. Although human follicles isolated from thawed–frozen tissue can develop to the antral stage following xenografting [70, 86, 87], there is a paucity of information regarding the ability of human follicles isolated from thawed tissue to survive in

culture. In 1997, Oktay *et al.* demonstrated that the viability on isolation of primordial follicles enzymatically isolated from thawed human cortical tissue is the same as that of primordial follicles isolated from fresh tissue [83], yet when cultured individually survival rates are extremely poor [18, 24]. Work in our laboratory in collaboration with Outi Hovatta's group in Stockholm is focusing on optimizing culture conditions to support cryopreserved tissue. Our preliminary results show that, following mechanical isolation, follicles harvested from thawed-vitrified or slow frozen fragments of human cortex cultured for 6 days are capable of further individual growth at a rate similar to those isolated from fresh tissue [51; Telfer and McLaughlin, unpublished observations]. Poor results observed in earlier studies [18, 24] may reflect the method of follicle isolation and that the effect of enzymatic isolation and premature loss of somatic cell contact impacts negatively on the ability of follicles isolated from thawed tissue to survive in vitro.

## Culture of human oocyte cumulus complexes

The ultimate aim of a system supporting follicle growth is to produce competent epigenetically normal oocytes. In order to achieve this, in-vitro grown human oocytes need to be matured in vitro. Maturation of oocytes already exists as a separate strategy and this is utilized routinely in human ART processes with varying degrees of success [27, 88]. As discussed earlier, achieving and sustaining oocyte growth is the major objective of any complete in-vitro development system as this is a size-specific indicator of the oocyte's ability to resume meiosis [11, 48, 49, 53]. Another consideration must be that the system should be capable of supporting the nuclear maturation and cytoplasmic differentiation of oocytes in vitro [11, 89], processes that in vivo require precise timing and hormonal regulation. It appears that, despite numerous studies and a plethora of media tested, no maturation protocol has emerged as demonstrably superior [90]. While 40–80% of immature human oocytes can successfully complete IVM and IVF giving rise to live births, the rate of maturation of immature oocytes lies well below that of oocytes from stimulated ovaries indicating that either the protocols are suboptimal or many of the harvested oocytes are intrinsically unable to undergo maturation. In-vitro grown oocytes may require a further period of growth within the cumulus complex before maturation (Figure 33.2), and oocyte growth within human complexes has been demonstrated in vitro [53]. While there are no culture systems established to support the in vitro development of human oocytes from the mid-growth stage, i.e. 90–95 μm, a live birth has been reported using bovine oocytes of this size [80]. The immature bovine oocytes required a period of up to 14 days in vitro to allow further oocye growth and development to support maturation [80]. This suggests that a similar system could be applied to human oocyte cumulus complexes in order to achieve oocyte diameters suitable to undergo current IVM protocols.

## Proposed multi-step culture system for human oocytes

The successes reported at each of the stages of human follicle development outlined above supports the theory that complete development of quiescent human primordial follicles could be achieved in vitro. Currently there are culture systems for humans and domestic species that have been designed to support specific stages of development, but there are significant gaps in oocyte development that have not yet been supported in vitro. Development of a complete multi-step culture system requires us to fit each of these culture systems together (Figure 33.2), and future research needs to focus on the processes necessary to bridge the current gaps that would allow continuous development from primordial to developmentally competent stages. Significant advances have been made towards achieving this, particularly with the development of a system that supports activation and early growth [6] and subsequent growth to antral stages [6, 7]. The challenge now is achieving the final steps of maturation (IVM) and fertilization (IVF) of these in vitro grown (IVG) oocytes without the need to culture whole antral follicles.

The stages required for a multi-step culture system are: (a) cortical strip culture to support initiation and growth of primordial follicles to pre-antral stages, followed by (b) isolated follicle culture to support development from pre-antral to antral stages (c) and then, subsequently, (d) a system to support later stages of oocyte growth and development without the very large antral follicles of humans and domestic species (Figure 33.2). This would involve: (1) improving growth of primordial follicles in vitro so that follicles develop to a stage at which they can then be isolated, allowing progression to the next culture step; (2) optimizing

conditions that enable the transition of these isolated follicles from pre-antral to antral stage of follicle development in vitro, since at present oocyte–somatic cell interactions are not fully supported at the antral transition; and (3) developing in vitro systems to support oocyte growth and subsequent maturation of oocyte–granulosa cell complexes taken from the in-vitro grown early antral follicles, hence removing the requirement to support large pre-ovulatory follicle growth in vitro.

## Summary

The achievement of complete mouse oocyte development in vitro has led to investigation into the potential of large mammal and human oocyte development within culture systems. The individual success of several keys stages of this process, i.e. initiation of primordial follicle growth, follicle differentiation, completion of oocyte growth and IVM have been encouraging, although a unified system incorporating all of these developmental milestones has yet to be defined for human follicles. Although significant progress has been made, much optimization is still required to routinely complete the in vitro development of the stages detailed above. Translation of any in-vitro human follicle growth system into a clinical setting will require rigorous testing to determine the normality and health of in-vitro grown oocytes prior to the application of IVF procedures. Basic research using rodent models suggest that oocyte development in vitro does not result in adverse developmental outcomes or long-term effects [91]. Whilst this is encouraging, it is also essential that good models, using large animal models, are rigorously tested before proceeding with translating human culture systems to a clinical setting.

Whilst the therapeutic potential of in-vitro grown human oocytes may not be imminently realized, the methodology itself is central to fertility preservation programs. The culture systems provide a reproducible and effective technique to assess the viability of cryopreserved cortical strips prior to transplantation. Furthermore, these systems allow many basic scientific questions regarding human oocyte development to be addressed and, as a consequence, factors and mechanisms involved in its regulation identified. The knowledge gained from these basic studies will facilitate the development of optimized culture systems, which could have the prospect of clinical application to restore fertility in young women currently storing tissue.

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Chapter 34

# Contributions of ovarian stromal cells to follicle culture

David J. Tagler, Lonnie D. Shea and Teresa K. Woodruff

## Introduction

In-vitro culture systems for ovarian follicles are enabling tools for advancing the study of folliculogenesis and the development of fertility preservation techniques. Folliculogenesis is a complex process regulated by endocrine, paracrine and autocrine factors, and can be difficult to study in vivo. In-vitro culture systems provide a controlled environment in which to investigate the mechanisms driving follicle development. The usage of these systems has produced significant discoveries about the influence of hormones, mechanics and extracellular matrix (ECM) proteins in folliculogenesis [1–6]. In addition to these fundamental observations, these culture systems are providing a foundation for the development of systems for fertility preservation for cancer survivors [7, 8]. The increase in survival rates for young women with cancer has prompted the need for fertility preservation techniques [9]. Lifesaving cancer treatments, such as chemotherapy and radiation, threaten fertility by diminishing the immature follicle pool and triggering early menopause. Current treatments include cryopreservation and transplantation of ovarian tissue, which incurs the risk of re-introducing cancer cells into the patient [10, 11]. The successful development of follicle culture systems could circumvent this risk, by allowing in vitro follicle maturation/fertilization to obtain fertilizable oocytes from immature follicles. Follicle culture systems have had some success; however, further developments are necessary to achieve the consistent growth of human follicles to produce fertilizable oocytes [12, 13].

Stromal tissue is essential for the development of the earliest stage follicles. The ovarian stroma contains interstitial–theca cells, neurons, blood vessels and macrophages. Secondary follicles can be grown individually using in-vitro culture systems; however, primordial and primary follicles are typically activated in organ culture-containing stromal components [13–18]. Hence, the stroma has a clear influence on follicle development. Stromal cells provide structural support and have complex bi-directional paracrine signaling with the follicle [19–22]. Moreover, it is widely hypothesized that stromal cells are recruited by the follicle and differentiate into theca cells [23–27]. Nevertheless, the specific mechanisms of action remain unclear. In-vitro culture systems that contain stromal cells may be an enabling tool for investigating the mechanisms by which stromal cells activate the early stage follicles and may ultimately be translated toward strategies for fertility preservation for cancer patients.

In this chapter, we discuss ovarian stromal cells and in-vitro follicle culture systems. Integrating stromal cells into current follicle culture systems will better simulate the natural ovarian microenvironment and could lead to the elucidation of the mechanisms by which these follicles are activated. In addition, stromal cell co-culture could improve early follicle growth and survival, which are essential for the successful translation of in-vitro culture systems to primate and human follicles. The current knowledge base for the culture of somatic and stromal cells is discussed, with an emphasis on three-dimensional culture systems using biomaterials. In addition, the potential and challenges of co-culture is discussed by drawing from recent work with co-culture systems in tissue engineering.

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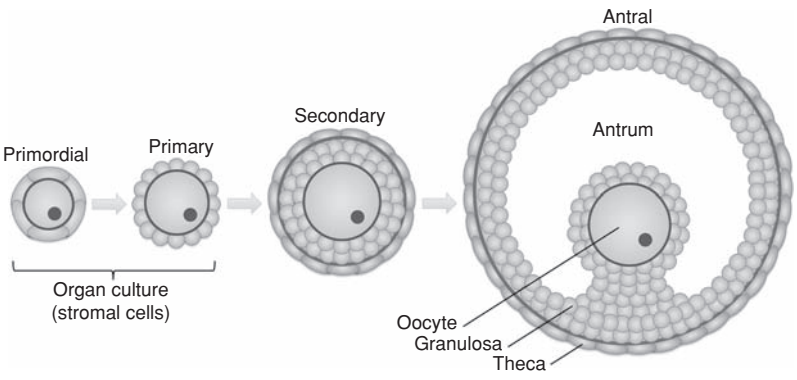


**Figure 34.1** Folliculogenesis. Primordial follicles develop to antral follicles, which are capable of producing fertilizable oocytes. Ovarian stromal cells are hypothesized to have significant roles in the activation of primordial follicles and the recruitment/differentiation of theca cells. Most follicle culture systems focus on secondary or multilayer follicles and produce antral follicles with fertilizable oocytes. Primordial and primary follicles do not activate or mature in vitro. The culture of these follicles is typically performed as an organ culture. See plate section for color version.

## Roles of ovarian stromal cells in follicle development

Folliculogenesis is the development process during which primordial follicles develop to Graafian follicles that ovulate fertilizable oocytes (Figure 34.1). For comprehensive reviews of folliculogenesis, please refer to references [28–30]. The follicle is the functional unit of the ovary and is composed of a germ cell (the oocyte) and layers of somatic cells (granulosa and theca cells). Primordial follicles are the most immature class and are found during embryonic (human) or immediately post-embryonic (murine) life. The oocyte within primordial follicles is arrested in the first meiotic prophase. Follicle activation from the non-replenishable ovarian reserve is a process that is not well understood. Selected primordial follicles are activated and form primary follicles, which have larger oocytes and a layer of cuboidal granulosa cells enclosed by a basement membrane. Subsequently, the granulosa cells proliferate and form several layers around the oocyte. At the same time, theca cells begin to surround the basement membrane of the follicle. These follicles are called secondary and multilayer follicles. Under the influence of follicle stimulating hormone (FSH), the granulosa cells proliferate and differentiate into cumulus (surround the oocyte) and mural (inside of basement membrane) granulosa cells. Likewise, the theca cells differentiate into theca interna (androgen secreting cells) and externa (connective and supportive tissue). The follicle increases in size and develops a fluid-filled cavity called an antrum. Under the influence of luteinizing hormone (LH), the oocytes in antral follicles complete the first meiosis cell division and then pause in the second meiotic metaphase. These oocytes are capable of fertilization. The overall goal of follicle culture systems is to reproduce this entire process in vitro.

Ovarian stromal cells may have significant roles in folliculogenesis, particularly in the activation of primordial follicles and the differentiation of theca cells. Stromal cells, which are similar in morphology to fibroblasts, make up the connective tissue throughout the ovary and surround follicles. Stromal cells are assumed to arise from a population of unspecialized mesenchymal stem cells [31]. The morphology of the stromal tissue varies between the cortex and medulla of the ovary [32]. In the cortex, the stromal cells are organized parallel to the surface and have a rounded structure. In the medulla, the cells exhibit random organization and have an elongated structure, and they are often referred to as interstitial or luteinized cells. The stromal cells in the medulla are believed to be further differentiated towards theca cells and have greater steroidogenic capacity than the stromal cells in the cortex [33]. Specific stromal cell markers have not yet been identified. Hence, stromal cells are often identified as mesenchymal cells that lack theca cell markers, such as the LH receptor (LHR), steroidogenic acute regulatory protein (*StAR*), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase (CYP17A1) [33–35]. Stromal cells signal via paracrine factors and influence early follicle development. For example, bone morphogenetic proteins 4 and 7 (BMP-4 and -7), secreted by stromal and/or theca cells have been identified as positive regulators of the primordial-to-primary follicle transition [36–38]. Moreover, it is widely hypothesized that stromal cells are recruited by the follicle via paracrine factors and differentiate into theca cells. This hypothesis was first proposed by Dubreuil in 1946 [23] and later reiterated by others [24–27]. To

date, a number of paracrine factors that regulate theca cell recruitment and differentiation have been identified, such as insulin-like growth factor (IGF-1), kit ligand (KL) and basic fibroblastic growth factor (bFGF) [22, 39, 40]. Nevertheless, the conclusive evidence for this hypothesis remains elusive. In-vitro culture systems of follicles and stromal cells may be an enabling tool to elucidate the functions of the ovarian stroma, signaling mechanisms and roles in follicle development.

## Studies investigating the roles of the ovarian stroma

### Follicle-to-stroma paracrine signaling

Early stromal cell experiments established paracrine signaling from the follicle to the stroma. In 1995, Magoffin and Magarelli demonstrated that granulosa cells of developing follicles secrete a signal that stimulates theca cell differentiation [19]. In this experiment, isolated theca–interstitial cells from rat ovaries were cultured in media conditioned by follicles and assayed for androgen secretion. The conditioned media was found to stimulate androgen secretion, which suggested the presence of a theca differentiation factor secreted by the follicle. Later, Magarelli *et al.* identified an increase in the mRNA expression of the LHR and various theca steroidogenic enzymes, including cholesterol side-chain cleavage (P450scc), 3β-HSD and CYP17A1 in response to this media [21]. Also, Magarelli *et al.* showed that this differentiation signal was both gonadotropin (FSH) and developmentally regulated, as only pre-antral follicles with 2–5 layers of granulosa cells produced the signal [21]. To date, the identity of this signal has not yet been purified; however, proteins in the range of 19–24 kD that interact synergistically may be responsible for this action [20]. In a series of candidate signaling studies, the combination of two granulosa produced peptides, IGF-I and KL, were found to increase androgen production and gene expression of androgenic factors in rat theca–interstitial cells [22]. Other potential regulating factors include growth differentiation factor-9 (GDF-9), activin, inhibin and follistatin [20, 41–46]. In sum, these experiments support the notion that follicle-to-stroma signaling exists.

Additional studies have demonstrated the effects of follicle-to-stroma signaling on theca cell recruitment, stromal cell proliferation and the primordial-to-primary follicle transition. Parott and Skinner treated ovary fragments and isolated stromal–interstitial cells with KL, which was hypothesized to be a "theca cell organizer" secreted by granulosa cells [39]. Kit ligand was found to significantly increase the percentage of theca cell layer thickness of primary follicles in organ culture. This result suggests KL helps to recruit theca cells from the stroma. Furthermore, KL was found to stimulate ovarian stromal cell proliferation in a dose-dependent manner. Treatment with KL did not affect stromal cell androstenedione or progesterone production. Hence, KL did not promote theca cell differentiation, which is consistent with research that has determined that theca cell differentiation is controlled by a synergy of multiple factors [20–22]. Later, Nilsson and Skinner identified the roles of KL and bFGF in the primordial-to-primary follicle transition [40]. The combination of these factors decreased the percentage of primordial follicles and increased the percentage of primary, pre-antral and antral follicles. Overall, these studies build upon the earlier work by identifying the possible roles of stromal cells in follicle development.

To further explore the effects of paracrine signaling, stromal cells have been co-cultured with granulosa and theca cells. Orisaka *et al.* developed a co-culture system that separates two cell populations via a collagen membrane, which permits the diffusion of factors smaller than 12.5 kD [33]. In these studies, bovine stromal cells from the cortex and medulla of the ovary were cultured with and without granulosa cells. Co-culture with granulosa cells increased the number of secreted lipid droplets, filopodia and mitochondria. Co-culture also stimulated androgen secretion in both cortex and medulla stromal cells. However, an increase in LH receptor mRNA was only observed in cortex stromal cells. Surprisingly, no increase in other theca markers was detected. While these data suggests theca cell differentiation, the evidence is not conclusive. No changes in the co-cultured granulosa cells were reported. Nevertheless, these experiments successfully demonstrated paracrine signaling from granulosa cells to the stromal cells in a co-culture system.

## Stroma-to-follicle paracrine signaling

In addition to paracrine signaling from the follicle to the stroma, signaling from the stroma to the follicle has been studied. The bone morphogenetic proteins BMP-4 and BMP-7, which are expressed by stromal

and/or theca cells, have been linked to the primordial-to-primary follicle transition. Lee *et al.* demonstrated that BMP-7 promotes the activation of primordial follicles in vivo [38, 47]. BMP-7 was injected into the ovarian bursa of rats, which produced a decrease in the number of primordial follicles and an increase in the number of primary, pre-antral and antral follicles. Nilsson *et al.* achieved similar results using in-vitro organ culture and BMP-4 [37]. Thus, these studies support the notion that stroma-to-follicle signaling exists.

## Organ culture and the activation of primordial follicles

Some of the most significant stromal cell experiments have been ovary organ culture experiments (*in situ*). These experiments culture follicles within thin fragments of ovarian stromal tissue. In 1996, Eppig and O'Brien achieved complete oocyte development in vitro using oocytes from primordial follicles of newborn mice [14]. This result was accomplished using a two-step strategy in which the ovaries of newborn mice were grown in organ culture for 8 days and then oocyte–granulosa cell complexes were isolated from the ovaries and cultured individually for an additional 14 days. Using this strategy, primordial follicles developed to produce mature oocytes. These oocytes were then fertilized in vitro and the resulting embryos were implanted to produce live offspring. When cultured individually outside of stromal tissue, primordial follicles rapidly lose their three-dimensional structure, pre-granulosa cells migrate away from the oocyte and oocyte extrusion/degeneration occurs [48]. Follicle culture in ovary organ fragments provides a complex support system that closely resembles the in-vivo ovary environment. Follicles maintain contact with the supporting stromal cells, which provide the local biochemical control pathways that trigger the activation of follicle growth [49]. Eppig and O'Brien's method was later improved and translated to follicles from larger animals, such as cow and sheep [15–18]. Recently, Telfer *et al.* used this method to activate primordial human follicles [13]. Primordial follicles were matured to secondary follicles in organ culture and then cultured individually to the early antral stage with the treatment of activin. Hence, these experiments motivate the need to integrate stromal cells into the current follicle culture system in order to achieve the activation of individual primordial and primary follicles in vitro.

## Three-dimensional culture systems for ovarian follicles

Most follicle culture systems have focused on isolated secondary/multilayer follicles or oocyte–granulosa cell complexes. Primordial and primary follicles do not mature when cultured individually in vitro. The majority of work has been completed using rodents due to the low cost and relatively short-growth period compared to larger animals. Follicle culture systems can be divided into two approaches: two-dimensional (flat) (Figure 34.2a) and three-dimensional (spherical) (Figure 34.2b). For detailed reviews of follicle culture systems see the following references [49–51]. In two-dimensional follicle culture systems, follicles are cultured on flat surfaces such as tissue culture plastic (polystyrene), collagen or polylysine. The first successful culture system using isolated mouse follicles was developed by Eppig in 1977 [52]. This system was later improved and achieved the birth of live offspring [14, 53, 54]. While two-dimensional culture systems have proved to be successful at producing mature oocytes in mice, this approach has proven difficult to replicate with follicles from large animals and humans. The unnatural geometry of two-dimensional culture disrupts cell–cell communication and causes the granulosa cells to break though the basement membrane, migrate away from the oocyte and attach to the two-dimensional surface [3, 48, 55, 56]. Thus, in order to culture larger follicles and achieve the goal of maturing human follicles in vitro, three-dimensional culture systems are necessary to properly support the developing follicle.

Three-dimensional follicle culture systems maintain the natural spherical geometry and cell–cell interactions of the follicle. Three-dimensional culture systems have utilized polylysine or collagen-coated substrates [55, 57], hydrophobic membranes [58], rotating walls and orbiting test tubes [59], mineral oil with daily follicle transfer [60], inverted culture [61, 62] and serum-free media [13, 63]. These systems have been successful at maintaining follicle geometry, preventing granulosa cell migration and minimizing attachment to the flat surface. Nevertheless, these systems still lack the ability to support large follicles for extended culture times. Fortunately, the application of biomaterials to follicle culture offers the potential to overcome these limitations and provide a true three-dimensional culture environment.

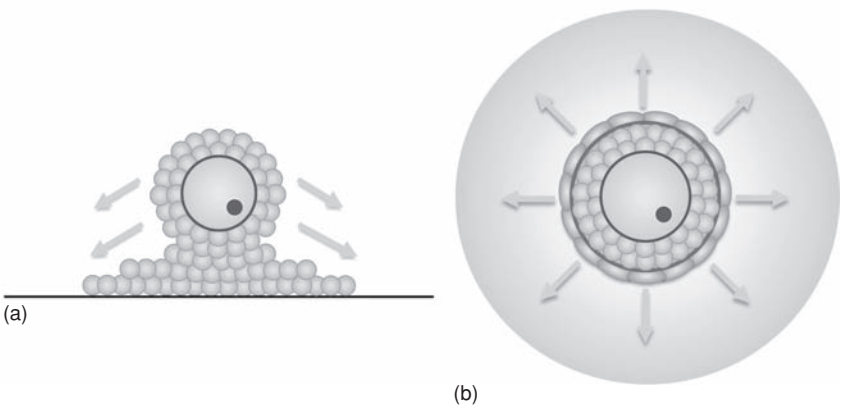


**Figure 34.2** In-vitro follicle culture systems. (a) In two-dimensional systems, follicles are cultured on flat surfaces such as tissue culture plastic (polystyrene). The unnatural geometry/mechanics of these systems disrupts cell–cell communication and causes the granulosa cells to break though the basement membrane, migrate away from the oocyte, and attach to the two-dimensional surface. Two-dimensional systems lack the ability to support large follicles for extended culture times. (b) In three-dimensional systems, follicles are cultured within biomaterial scaffolds, such as alginate. These systems maintain the natural spherical geometry and cell–cell interactions of the follicle. See plate section for color version.

Three-dimensional biomaterial scaffolds mimic in vivo cellular microenvironments better than flat two-dimensional surfaces [64, 65]. The usage of biomaterial scaffolds has demonstrated the effect of geometry and mechanics on cell survival, proliferation, migration, gene expression and differentiation. For example, Bissell and coworkers demonstrated that human mammary epithelial cells display a spread phenotype when cultured on a two-dimensional surface, yet form normal acinar structures when cultured in a three-dimensional environment [66, 67]. Moreover, Tanaka *et al.* found that enhanced chondrogenesis resulted from the three-dimensional culture of embryonic stem cells compared to flat monolayer culture [68]. In addition to geometry, mechanics has a substantial effect on cell behavior. For example, Engler *et al.* identified that stem cell lineage was controlled, in part, by scaffold elasticity [69]. Mesenchymal stem cells were directed to neurogenic, myogenic and osteogenic lineages using soft, stiff and rigid matrices. Hence, these studies have established the need to provide the proper environment for cell culture. This task has been accomplished via the use of biomaterials that permit the modification of physical properties. Thus, biomaterials have provided an enabling tool to mimic in vivo environments.

Highly water-soluble polymer networks, called hydrogels, have been used as biomaterial scaffolds for follicle culture. Hydrogels support natural follicle growth by mimicking the natural stromal microenvironment of the ovary. Early hydrogels for follicle culture employed collagen [70–74], which is an extracellular matrix protein that is prominent throughout the ovary [75]. Torrance *et al.* demonstrated the growth and survival of primary mouse follicles in collagen hydrogels, but did not achieve antrum formation [71]. Later, Hirao *et al.* produced mature oocytes from pre-antral pig follicles [74], and Sharma *et al.* achieved antral follicles from pre-antral buffalo follicles [72]. In addition to collagen, alginate, a common tissue engineering hydrogel, has shown great promise as a scaffold for follicle culture [1, 3–6, 12, 76–78]. It is a naturally derived polysaccharide isolated from brown algae. Alginate is a block copolymer composed of blocks of (1–4)-linked β-D-mannuronic acid (M units) and its C-5 epimer α-L-guluronic acid (G units) [79]. These blocks can be either similar or alternating. Alginate chains can be crosslinked with divalent cations, such as $Ca^{2+}$. As a result, alginate avoids the use of harmful chemicals, ultraviolet light or temperatures to crosslink the polymer network [50]. Crosslinking occurs via interaction of the carboxylic acid functional groups in the G-blocks. This crosslinking leads to the formation of a gel network while retaining cell viability and the cellular interactions within the follicle. Due to its hydrophilic nature, alginate discourages protein adsorption and cell attachment. Thus, cells are unable to specifically interact or bind with alginate. In order to overcome this limitation, alginate can be covalently linked to cell-adhesion molecules, such as RGD (Arg-Gly-Asp)

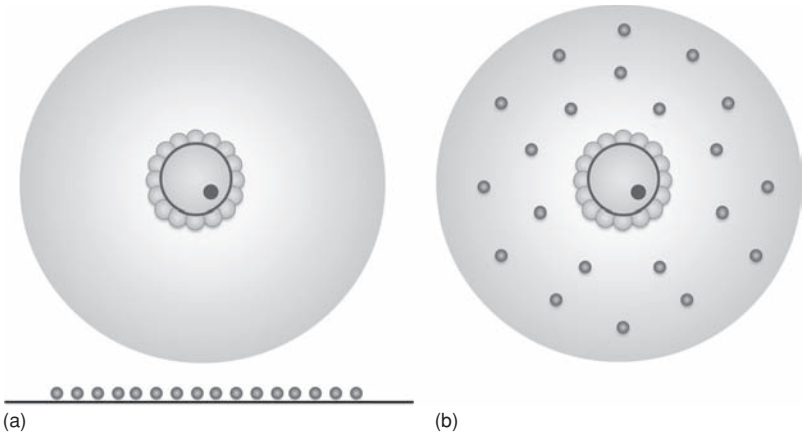


**Figure 34.3** Stromal cell co-culture approaches. (a) Stromal cells can be cultured separately on a flat surface below the encapsulated follicle. This set up allows for paracrine signaling between the follicle and the stromal cells. (b) Stromal cells can be encapsulated inside the biomaterial scaffold with the follicle. This set up allows for paracrine signaling as well as cell–cell attachment and interaction with secreted extracellular matrix proteins. See plate section for color version.

peptide ligands, via its carboxylic acid functional group and carbodiimide chemistry [80]. In addition, alginate can be degraded with alginate lyase, an alginate specific enzyme, in order to safely remove the follicle from the hydrogel. Hence, due to its inert nature, gentle gelatin and modularity, alginate was a top candidate for follicle culture.

Alginate is a successful encapsulating matrix for follicle culture. Experimentally, isolated follicles are suspended within drops of alginate, which are subsequently crosslinked in a $Ca^{2+}$ solution. Each resulting alginate bead is then transferred to an individual well in a standard cell culture plate containing growth media. Alginate hydrogels have been shown to maintain the natural three-dimensional morphology of the developing follicle [3]. This culture system has demonstrated the successful maturation of secondary to antral follicles with mice, primate and human follicles [1, 4–6, 12, 76–78]. Follicles grow, develop antrums and produce meiotically competent oocytes. These oocytes have been successfully fertilized in vitro and implanted into mice, which has yielded healthy, fertile offspring [76]. Follicle growth was found to be a strongest function of alginate concentration or matrix mechanical properties. Permissive environments (low concentrations of alginate) supported the most rapid follicle growth and highest rates of fertilization [4, 5, 50]. In addition, the incorporation of extracellular matrix (ECM) proteins, such as collagen, fibronectin and laminin, into the alginate matrix has been found to improve follicle growth and oocyte quality [3]. This culture system has also been used in combination with ovary organ culture to produce mature oocytes from primordial follicles [15]. Furthermore, alginate has been used to culture human follicles after cryopreservation [81]. Hence, alginate has proven to be a powerful tool for the advancement of follicle culture.

Additional functionalities are being incorporated into the alginate system to enhance follicle development. A fibrin-alginate interpenetrating network (FA-IPN) was developed to provide dynamic cell-responsive mechanical properties to the culture system [77]. Fibrin, the natural polymer involved in blood clotting, and alginate are formulated simultaneously into a single hydrogel. As the follicle grows, proteases are secreted to degrade the fibrin, leaving only the non-degradable alginate matrix to support the follicle. Fibrin alone was not successful in promoting follicle development. This FA combination produces a more permissive environment than can be achieved with alginate alone. The rate of meiotically competent oocytes produced was 82%, which is significantly higher than alginate alone or any other reported in-vitro culture system. By promoting interaction with the encapsulating matrix, degradable hydrogels better mimic the natural ovary microenvironment. Future follicle culture systems will build upon this example in order to further improve oocyte quality and survival, which is essential for the complete translation to primate and human follicles.

## Utility of co-culture for ovarian follicle development

The next logical step in the development of follicle culture systems is the integration or co-culture of stromal cells (Figure 34.3). As demonstrated via stromal cell and ovary organ culture experiments,

stromal cells have a significant role in the activation of primordial/primary follicles and theca cell recruitment/differentiation. The paracrine signaling involved in these processes is hypothesized to be a complex time-dependent synergy of unidentified factors [20]. Cell–cell contact could also have an important role in these processes. Accordingly, the simple addition of candidate hormones and growth factors to the culture media has not yet achieved success. Until the mechanisms of these processes is elucidated, follicle culture systems could attempt to incorporate stromal cells in order to potentially culture smaller follicles and improve growth, survival and oocyte quality.

While only a few co-culture studies with stromal cells have been conducted, these experiments demonstrate positive results and motivate further investigation. Osborn *et al.* showed that the presence of stromal cells around isolated primordial follicles improved initial culture success [82]. Building upon this observation, Itoh and Hoshi co-cultured small pre-antral (primary and secondary) bovine follicles with ovarian mesenchymal cells, granulosa cells and skin fibroblasts for 30 days [83]. Compared to the non-co-culture controls, follicle viability was significantly increased in all three co-cultures (18.6, 17.1 and 10.0%, respectively) and follicle growth was significantly increased in the mesenchymal (15.4%) and fibroblast (30.0%) co-cultures. In a similar fashion, Wu *et al.* co-cultured pre-antral pig follicles with different follicular cells [84]. In contrast to Itoh and Hoshi's results, Wu *et al.* found that small pre-antral follicle growth and survival was inhibited by co-culture with multiple follicles (with or without oocytes). The growth and survival of these follicles was only enhanced when co-cultured with cumulus cells from antral follicles >3 mm in diameter. Moreover, Ramesh *et al.* co-cultured buffalo pre-antral follicles with different somatic cells (cumulus, granulosa, mesenchymal and epithelial) [85]. Co-culture with cumulus, granulosa and mesenchymal cells resulted in better development, growth rate and survival than the control and epithelial cells. Maximum growth and survival was achieved via co-culture with cumulus cells, which supports Wu's results. Therefore, these studies clearly demonstrate the utility and effect of co-culture. Nevertheless, these co-culture experiments have not yet been conducted in the current state-of-art three-dimensional culture systems with optimized culture media. Hence, the impact of co-culture could be significantly enhanced by integrating stromal cells into current three-dimensional culture systems.

The integration of stromal cells into three-dimensional culture systems is possible, but will require some adaptations of the culture system. The challenge will be to develop a hydrogel matrix that promotes stromal cell survival within the constraints of follicle growth. Unfortunately, stromal cells cannot be added directly to the alginate follicle culture system without considerable modifications. Alginate does not support cell attachment. However, this can be accomplished by covalently linking alginate to full extracellular matrix proteins, such as collagen, fibronectin and laminin or small peptides sequences from these proteins. Without these attachment sites the cells will undergo apoptosis or cell death. Alternatively, other biomaterials such as collagen, Matrigel or polyethylene glycol (PEG) could be employed for co-culture. Unquestionably, the modifications needed for stromal cell culture will also influence follicle growth. Hence, it will be difficult to find overlapping culture conditions for both stromal cells and follicles. For example, the concentration of stromal cells must be fine-tuned to allow adequate paracrine signaling while not starving the follicle of oxygen or nutrients. Thus, developing a three-dimensional co-culture system will be challenging, but it is definitely an obtainable goal.

## Examples of co-culture success in tissue engineering

In considering the utility of co-culture for ovarian follicle development, we considered the success that has been achieved for other biological systems. Perhaps the foremost example of co-culture success in tissue engineering has been the development of an artificial bladder by Atala *et al.* [86]. Urothelial and muscle cells were grown in culture and then seeded on a biodegradable bladder-shaped scaffold made of collagen. The exterior surface of the scaffold was seeded with the smooth muscle cells and the interior was seeded with urothelial cells. These artificial bladders were implanted into seven patients with myelomeningocele (spina bifida) and either high blood pressure or poorly compliant bladders. After surgery, bladder biopsies showed an adequate structural architecture and phenotype. These implants have been shown to be functional and durable over a period of years [86]. This example demonstrates that co-culturing multiple cell types via

biomaterial scaffolds is an effective strategy to recapitulate natural cellular environments and restore cell–cell communication.

Co-culture has also been successfully applied to numerous cell types including skin, cartilage, bone, liver, blood, nerve and stem cells [87–91]. These experiments have demonstrated that co-culture can promote or inhibit differentiation of one or both cell types. No correlation has yet been found between co-culture methods, cell types or differentiation. Nevertheless, co-culture has proven to be effective at improving the survival, proliferation and function of cells. For example, Houchin-Ray *et al*. developed a co-culture model consisting of primary neurons and accessory cells in order to promote and direct neurite outgrowth [92, 93]. Co-culture promoted neuron survival and neurite extension. With respect to reproductive technology, co-culture has been applied to in vitro maturation (IVM) and in vitro fertilization (IVF). Oocytes and developing embryos have been co-cultured with various feeder cells including oviduct endothelial, endometrial epithelial, fallopian tube ampullary, cumulus, granulosa and non-human cells such as the Vero cell line. While controversial, these experiments suggest that co-culture may improve IVM and IVF results [94–97]. Moreover, the undifferentiated state of embryonic stem cells has been maintained via co-culture with feeder cells such as fibroblasts [98, 99]. In sum, these examples have demonstrated the utility of co-culture, which potentially could have similar impacts in follicle culture.

## Conclusion

The co-culture of ovarian stromal cells and follicles is another step in the evolution of three-dimensional follicle culture systems. Stromal cell and ovary organ culture experiments have established the significant role of stromal cells in primordial/primary follicle activation and theca cell recruitment/differentiation. Hence, co-culture has the potential to activate individual primordial/primary follicles, which do not mature in any in-vitro culture system and improve growth, survival and oocyte quality. Current follicle culture systems that utilize biomaterials, such as alginate, provide three-dimensional scaffolds that maintain the natural geometry of the follicle and could enable the translation to follicles of larger animals and humans. These culture systems allows for the straightforward integration of stromal cells, which will help recapitulate the natural ovarian environment. Accomplishing this task will ensure that follicle culture systems continue to advance the study of folliculogenesis and the development of fertility preservation techniques.

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Chapter 35

# In vitro maturation of GV oocytes

M. De Vos and J. Smitz

## Introduction

Success rates of assisted reproductive technology (ART) have tremendously improved over the past three decades. Nevertheless, controlled ovarian stimulation protocols are still associated with a substantial risk of ovarian hyperstimulation syndrome (OHSS) which, in its severe form, may result in significant morbidity and even mortality [1]. The overall incidence of severe OHSS is <2% [2], but in women with polycystic ovary syndrome (PCOS), who represent up to 30% of women eligible for ART [3], the risk of OHSS is higher. These patients are therefore at increased risk of having the embryo transfer cancelled. Strategies to prevent OHSS in patients with a high response to gonadotropins include the administration of a gonadotropin-releasing hormone (GnRH) agonist instead of human chorionic gonadotropin (hCG) to induce the final oocyte maturation [4] and in vitro maturation (IVM) of oocytes. In vitro maturation avoids the risks and side effects of conventional ovarian hormonal stimulation because it involves retrieving immature oocytes from unstimulated or minimally stimulated ovaries. After immature oocyte collection, these oocytes are cultured, matured and fertilized in vitro. Selected embryos are then transferred to an adequately primed endometrium or, alternatively, are cryopreserved and subsequently thawed or warmed in a natural or artificial cycle.

The first successful pregnancy and birth from IVM in human was described more than 15 years ago. In spite of technical advances since then to the IVM protocol and improvements of the maturation method and culture media, implantation rates do not exceed 15%, they rather stagger at 10%. This often necessitates the transfer of more than one embryo, which in young women may increase the risk of multiple pregnancies. It also interferes with law restrictions in a number of countries where multiple embryo transfer precludes ART cost reimbursement by the government. Lower pregnancy rates in comparison with conventional ART, as well as concerns about the genetic health of IVM oocytes [5] and the long-term health of embryos, fetuses and children born after IVM, still preclude a more general acceptance of IVM in ART centers. The causes and mechanisms of the lower implantation rates of IVM-derived embryos are still largely unknown, although they are almost certainly linked to a lack of complete cytoplasmic maturation [6] and to a suboptimal endometrial receptivity associated with IVM cycles. There is a need to optimize IVM culture media [7], to standardize the IVM treatment protocol with regard to the use of estradiol and/or gonadotropins to prime the follicles and the endometrium [8], the aspiration technique and the IVM timings [9].

There is evidence that oocyte maturation, fertilization rates and blastocyst production are compromised when compared with in-vivo matured oocytes [6], possibly because of a dissynchronous nuclear and cytoplasmic maturation. Promising technologies have emerged in recent years to improve maturation rates and developmental competence of resulting embryos, based on methods that can compensate for the reduced maturation time (24–36 h) that oocytes undergo spontaneously when aspirated out of follicles, compared to the situation where they would remain within the ovary [10]. Recent IVM culture system modifications include substances that increase the cyclic adenosine monophosphate (cAMP) levels in the oocyte environment, which allows for a more physiological cascade of IVM triggering [11].

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This chapter will summarize the scope of IVM as a stand-alone ART and aims to contribute to the discussion of how IVM can become a valuable alternative to conventional ovarian stimulation.

## The emergence of IVM

The primordial follicle pool of the ovaries harbors oocytes that are arrested at prophase stage I of meiosis. The majority of these follicles become apoptotic; only a few follicles grow beyond the antral stage, at which time the oocyte continues to mature until ovulation. The molecular events that drive and regulate the process of oocyte maturation are not fully understood, although follicle stimulating hormone (FSH) is thought to play a major role in influencing the maturation process. After selection of the dominant follicle, this follicle becomes FSH dependent. The increasing estrogen levels from the pre-ovulatory follicle induce a luteinizing hormone (LH) rise, which in turn induces a cascade of secondary factors synthetized in the mural follicle cells that lead to breakdown of the germinal vesicle (GV) in the oocyte [12]. The oocyte proceeds from meiotic metaphase I to telophase I, which is associated with extrusion of the first polar body. The full meiotic progress does not only occur after the LH surge, but also when the oocytes become detached from their follicular environment. Historical observations by Pincus and Enzmann in 1935 showed that immature oocytes have the ability to resume meiosis spontaneously when they are removed from the follicle [13]. Edwards *et al.* confirmed this concept by showing that that they reach metaphase I approximately 28–35 h after being released from the follicle, that they extrude the first polar body after being in culture between 36 and 43 h and that they can be fertilized [14].

In vitro maturaton has a number of important advantages over in vitro fertilization (IVF). Ovarian hormonal stimulation is either limited or even absent, which eliminates the risk of OHSS. Furthermore, the immediate side effects of the ovarian stimulation drugs (i.e. nausea, vomiting, breast tenderness, abdominal swelling and mood swings) are avoided. With IVM there is no risk of premature luteinization and therefore the IVM protocol requires less meticulous follow-up and reduces the number of visits during the treatment cycle. Finally, IVM is more affordable for the patients because the incurred medication costs are significantly reduced. All of the above make IVM a "patient friendly" protocol in ART.

Cha *et al.* obtained the first IVM pregnancy in 1991 [15]. They had used the immature human oocytes retrieved during gynecological operations in an oocyte donation program. The first IVM pregnancy with a patient's own oocytes was achieved in 1994 and was obtained by Trounson *et al.* [16]. However, pregnancy rates after IVM in those years were disappointingly low, until Chian *et al.* introduced hCG priming for IVM in PCOS patients, 36 h prior to the oocyte retrieval [17]. They reported implantation rates of 32% and clinical pregnancy rates of 40%, which marked an important step forward [17]. During the following years the same group achieved clinical pregnancy rates of up to 54% after IVM in unstimulated cycles [18]. However, such high pregnancy rates were obtained after the transfer of 3–4 embryos irrespective of the age of the patient.

In-vitro maturation pregnancy rates have consistently been lower than IVF pregnancy rates after conventional ovarian stimulation [19]. The reduced success rates have been attributed to the asynchrony in the cytoplasmic and nuclear maturation of the oocyte as well as to an inferior endometrial thickness and receptivity [20]. Also, the final number of matured oocytes obtained in unstimulated cycles followed by IVM is relatively low as compared to the number of mature oocytes obtained in conventional stimulated cycles. Therefore, increasing the pregnancy and the live birth rates in IVM treatment has become a major focus of clinical and scientific research. A number of studies have focused on improving in-vitro culture media, whereas other studies have been designed to improve the quality and quantity of oocytes and the quality of the endometrium.

Whereas in the early years IVM was advocated as a particularly suitable technique for young women with a high antral follicle count, current and future improvements to the technology should contribute to its use in a wider scope of applications, including women with poor ovarian reserve, oocyte donors and fertility preservation medicine.

## Technical aspects of IVM

In patients with PCOS, a course of oral or vaginal progesterone to induce a withdrawal bleed is often prescribed [21]. However, whether this practice is truly necessary prior to the start of an IVM cycle remains

to be verified. After a baseline ultrasound scan to rule out the presence of cysts or other pathology, serial ultrasound scans are scheduled to assess the growth of the antral follicles and the thickness of the endometrium. Once the endometrial thickness has reached a minimum of 6 mm and the largest follicle is 10–12 mm, most centers administer hCG. The timing of egg retrieval has been shown to be important, as egg retrieval 38 h after hCG trigger instead of 35 h appears to yield an improved maturation rate in unstimulated cycles [22].

There have been multiple published reports with regard to the selection of the optimal day for egg retrieval based on the follicular diameter. When the leading follicle is larger than 13 mm, it has been demonstrated that fewer oocytes are retrieved, the fertilization rate is lower and fewer embryos are generated [23, 24], at least in unstimulated ovaries. On a similar note, Cobo *et al.* achieved higher rates of development to the blastocyst stage when the dominant follicle is smaller than 10 mm [25]. These reports may suggest the existence of a so-called dominant negative effect, in that large follicles may compromise the developmental potential of the oocytes in the smaller antral follicles, although this has not been confirmed by some authors [26, 27].

There is published evidence that the embryological and clinical outcome is improved in hCG primed IVM cycles with in vivo matured oocyte(s) as compared to cycles with only immature oocytes at pick-up [28, 29]. Retrieval of at least one in-vivo matured oocyte is more likely in those cycles where the leading follicle has reached a diameter of 10–12 mm before oocyte retrieval.

## How to improve implantation rates in IVM?

### Improving in-vitro culture techniques

The nuclear maturation through meiosis I and II is a prerequisite for successful oocyte maturation. Cytoplasmic maturation is equally important and includes relocation of organelles, synthesis and modification of proteins and mRNAs, and regulation of biochemical processes that support subsequent fertilization and embryonic development [30]. Regulation of oocyte maturation in vivo involves complex signaling pathways that occur in the microenvironment of the maturing oocyte. The oocyte and cumulus cells communicate through gap junctions [18] that allow passage of regulatory molecules and growth factors. The oocyte is in meiotic arrest until meiotic progress is triggered. In vivo, maturation is triggered by the endogenous LH surge and mediated by growth factors, such as epidermal growth factor (EGF) family members amphiregulin, epiregulin and beta-cellulin [12]. In vitro, oocyte maturation occurs spontaneously when the oocyte is removed from the follicular environment that inhibits meiotic progression [31]. When immature oocytes are removed from small antral follicles, meiotic resumption will occur precociously, i.e. before completion of cytoplasmic maturation. Therefore, the timing of resumption of meiosis is important in oocyte maturation. To solve this problem for IVM systems, some authors suggest delaying spontaneous nuclear maturation while promoting development of the cytoplasm at the same time [30]. The intracellular messenger molecule cAMP plays a significant role in the regulation of mammalian oocyte maturation [32]. High levels of cAMP and cAMP analogues prevent meiotic resumption [33]. Spontaneous oocyte maturation in vitro can be inhibited or delayed by increasing the cAMP level within the cumulus–oocyte complex (COC) environment by adding any of the following substances to the media: (1) cAMP analogues such as dibutyryl cAMP; (2) activators of adenylate cyclase, such as FSH, forskolin or invasive adenylate cyclise; and (3) phosphodieserase (PDE) inhibitors, such as the non-specific inhibitor IBMX, the PDE type 4-specific inhibitor rolipram or the PDE type 3-specific inhibitors milrinone, cilostamide or Org9935 [33]. These agents delay GV breakdown and simultaneously increase the extent and prolong the duration of oocyte–cumulus cell (CC) gap-junction communication during the meiotic resumption phase [33–35], which in turn extends the exchange of regulatory factors and metabolites between the oocyte and the cumulus cells [36]. Although treating oocytes in vitro with the addition of these cAMP elevating agents prevents meiotic resumption of mouse [32] and human oocytes [37], a beneficial effect on subsequent oocyte developmental potential in human and ensuing pregnancy rates with any of these agents has not yet been demonstrated. Recent controlled prospective studies in the bovine, porcine and mouse model have shown a clear improvement on blastocyst development rate (porcine, bovine) and implantation rates (mouse) in comparison to the conventional methods for doing IVM [11, 34, 38, 39].

Also, premature interruption of the gap-junction communication between the cumulus cells and the oocyte may compromise the developmental competence of the oocyte. Although IVM rate and progression to the embryonic cleavage stage may not be affected by absence of cumulus cells during maturation, the development to the blastocyst stage is significantly reduced in cumulus-free oocytes as compared to cumulus-intact oocytes [40].

## Hormonal priming

The introduction of hCG administration prior to immature oocyte retrieval significantly improved maturation, fertilization and pregnancy rates [17, 41, 42], although some authors did not find a beneficial effect of hCG priming with regard to the number of oocytes retrieved, maturation, fertilization or cleavage rates [33]. The exact mechanism by which hCG exerts an effect on small follicles is still unclear. At least there may be a technical advantage to its administration, in that the CC expansion of the oocytes secondary to hCG may facilitate detachment of the COC from the follicle during the aspiration. This in turn may improve the oocyte yield in hCG-primed IVM cycles compared to non-hCG primed IVM cycles. Priming with hCG may also increase the likelihood to obtain some in-vivo matured oocytes at retrieval, which is associated with improved blastocyst development rates, and explain the better implantation rates and clinical pregnancy rates.

## FSH priming

Compared to the use of hCG, the value of FSH priming in IVM cycles is more controversial. Follicle stimulating hormone (FSH) priming would lead to a technically easier oocyte retrieval, higher E2 levels and improved maturational competence of the oocytes, as well as improved endometrial priming [43].

In normo-ovulatory patients, Wynn *et al.* showed that administration of FSH during the early follicular phase is associated with a higher oocyte maturation rate [43], whereas others reported that FSH priming had no apparent effect on oocyte developmental competence [44, 45]. Suikkari *et al.* investigated the influence of low dose FSH priming in normo-ovulatory women starting from the late luteal phase and came to similar conclusions [46].

Conflicting results were reached in women with anovulatory cycles; Mikkelsen and Lindenberg demonstrated improved implantation and clinical pregnancy rates in FSH-primed cycles without additional hCG administration [47], whereas Lin *et al.*, who prospectively compared FSH-priming with hCG administration versus hCG alone, found no additional benefit of FSH-priming in those cases where FSH and hCG were both administered [48]. In a large randomized study encompassing 400 women, Fadini *et al.* demonstrated that FSH-priming with 150 IU/day FSH for 3 days from day 3 of the cycle plus hCG priming led to significantly improved maturation rates, implantation rates and clinical pregnancy rates, whereas FSH priming and hCG priming alone showed no significant effects on clinical outcome [49].

## Preparation of the endometrium

One of the major reasons why implantation rates have remained low compared with conventional IVF treatment is the insufficient development of the endometrium during IVM cycles before embryo transfer [23]. In conventional IVF cycles, the endogenous production of estradiol by the ovaries secondary to exogenous gonadotropin stimulation is much higher and allows for adequate endometrial proliferation on the condition that progesterone supplementation is provided in the luteal phase [50, 51]. But in endometrium preparation for IVM there is a need for estrogen supplementation to improve endometrial thickness, because of the shortened follicular phase of IVM cycles. As a result of the dissynchrony between the endometrial development and the embryonic development in IVM cycles, some centers prefer to freeze the embryos and to perform a thawed embryo transfer in an artificial or a natural cycle [52]. In IVM cycles with a thin endometrium, a recent retrospective study showed that both low dose human menopausal gonadotropin (HMG) and micronized 17β-estradiol supplementation significantly improve endometrial thickness, and that low dose hMG results in larger follicles and a greater number of in-vivo matured oocytes [53]. Nevertheless, there is a need for adequately powered prospective studies to investigate the optimal protocol for endometrial priming in IVM cycles and to correlate endometrial thickness in these cycles with endometrial receptivity at the time of embryo transfer, in normo-ovulatory women and in women with PCOS.

### Metformin

Metformin improves insulin sensitivity, lowers serum LH, total and free testosterone concentrations, and causes an elevation in serum FSH and sex-hormone binding globulin levels in obese women with PCOS [54]. Significantly higher implantation and clinical pregnancy rates were obtained in a series of 56 metformin pre-treated clomiphene-resistant PCOS patients undergoing IVM compared to the controls [55], suggesting that metformin may improve IVM outcome through optimization of the oocyte microenvironment, although the mechanism by which this occurs needs to be elucidated.

## Patient selection for IVM

A high yield of oocytes is a strong predictor of pregnancy following IVM treatment [56]. Although there is sufficient published evidence regarding the value of ovarian reserve tests (ORTs), including antral follicle count, anti-Müllerian hormone (AMH) and early-follicular-phase blood levels, in predicting poor ovarian response [57], the potential of these tests to predict a high response to ovarian stimulation is only modest. Women with PCOS or PCOS-like ovaries and those with a high antral follicle count (AFC) are undoubtedly the most suitable candidates to undergo IVM treatment [21]. The number of immature oocytes retrieved at egg collection is correlated with the AFC, the ovarian volume and the peak ovarian stromal velocity measured by Doppler ultrasound during the early follicular phase [56]. In view of the correlation between AFC and age [58], a low AFC, such as expected in the older age group, will be associated with lower implantation and clinical pregnancy rates, although advancing age itself also contributes to poorer oocyte quality. Therefore, the majority of IVM pregnancies in the literature have been reported in women with PCOS or PCOS-like ovaries and in women below 35 years of age [59]. In selected oocyte donors with high AFC, IVM is a promising alternative to a conventionally stimulated cycle, particularly in those donors who have concerns about short-term side effects of the ovarian stimulation or long-term health implications of the stimulation hormones. Furthermore, endometrial quality is not an issue in oocyte donors. Holzer *et al.* reported a clinical pregnancy rate of 50% (6/12) in an IVM oocyte donation program where the donors had PCOS or PCOS-like ovaries [60].

In normo-ovulatory women, natural-cycle IVF combined with immature oocyte retrieval followed by IVM can also lead to reasonable pregnancy and implantation rates. In a recently published series of 151 treatment cycles of natural cycle IVF, combined by IVM, a clinical pregnancy rate of 40.4% has been described [61].

Finally, IVM and oocyte or embryo cryopreservation have been presented as an attractive method of fertility preservation for cancer patients who need imminent cytotoxic treatment and do not have sufficient time to undergo conventional ovarian stimulation. Retrieval of immature oocytes from excised ovarian tissue was first reported by Revel *et al.* [62]. In a recently published series of four consecutive patients who underwent retrieval of immature oocytes from the antral follicles of the excised ovarian tissue, eight mature oocytes were eventually vitrified [63]. In cases of important time pressure before the start of gonadotoxic treatment and when the patient declines ovarian tissue cryopreservation, egg collection followed by IVM can also be applied during the luteal phase of a natural cycle [64]. Patients with hormone sensitive tumors are also excellent candidates for IVM. In a series of 20 women who underwent ICSI of vitrified–thawed in-vitro matured oocytes, a 20% pregnancy rate was reported [65].

## IVM as a rescue in case of threatening OHSS

When a high risk of OHSS emerges during conventional ovarian stimulation, the treatment cycle can be rescued by final oocyte maturation triggering occuring with a GnRH agonist, as opposed to triggering with hCG. This rescue mechanism was first reported in 2000 by Itskovitz-Eldor *et al.* [66], but had disappointing reproductive outcomes due to suboptimal luteal phase support. It was also known that without an hCG injection the oocyte recovery rate from follicles >12 mm diameter is suboptimal in comparison to retrievals from small and medium-size follicles.

Using adapted regimens of luteal support, Engmann *et al.* and Humaidan *et al.* independently reported the avoidance of OHSS while comparably good pregnancy rates were achieved [4, 67]. As an alternative, cancellation of further ovarian stimulation can be proposed, followed by retrieval of the immature oocytes, IVM, subsequent fertilization

by ICSI and then embryo transfer. However, immature oocytes obtained from conventionally stimulated ovaries have a lower fertilization rate [68], and the resulting embryos have high incidence of non-cleavage and chromosomal anomalies [69]. These observations appear to indicate that oocytes that remain at the GV stage after conventional ovarian stimulation are of inferior quality or have a rare intrinsic follicular or oocyte-specific defect.

## IVM outcome

As compared to conventional ART protocols, IVM is patient-friendly and IVM success rates have been increasing in the past decade. In general, implantation rates of 10–15% and clinical pregnancy rates of 30–35% have been reported in women with PCOS and PCOS-like ovaries, although usually three times more embryos are transferred than in the conventional ART setting.

All ART pregnancies are associated with an increased risk of multiple pregnancy, cesarean delivery and congenital abnormality. Babies born to date (presumably 1500–2000 children) following IVM treatment showed no increased risk of congenital abnormality or adverse perinatal outcome over that already accepted for IVF or intracytoplasmic sperm injection (ICSI) [70]. Nevertheless, a higher rate of clinical miscarriage has been observed following IVM as compared to IVF and ICSI. This appears to be related to the patient characteristics of the treatment group, i.e. patients with PCOS, rather than to the IVM procedure itself since the miscarriage rates following IVM and IVF/ICSI were comparable in patients with PCOS [70]. There is currently no strong evidence that IVM technology might have a higher risk to the development of epigenetic pathologies, as the GV oocytes studied were "left-overs" from normal superovulated patients [71, 72]. These oocytes were arrested in meiosis despite a normal FSH and hCG stimulation and might have been intrinsically compromised [69]. To the contrary, recent experiments in mouse models have demonstrated that in vivo superovulation with a higher dose of pregnant mare's serum gonadotropin (PMSG) induced methylation errors in some imprinted genes [73]. Hence, absent or minimal ovasian stimulation in an IVM procedure might obviate the increased risk of epigenetic abnormalities, as seen with high doses of gonadotropins. As for the risk of in vitro culture per se, it was demonstrated that in a long-term mouse follicle culture model the primary imprinting pattern for Snrpn, Peg3, H19 and Mest was completely normal [74, 75, 76]. More research needs to be done in animal models and in donated human oocytes to evaluate further, on a larger number of oocytes, what are the real risks of superovulation and IVM on the acquisition of imprints in growing oocytes and on the maternal-effect gene products subsequently required for imprinting maintainance.

## IVM and fertility preservation

Advances in oncological treatments have significantly improved the survival rates for different malignant tumors, including hematological pathologies, rectum and colon cancer and early-onset breast cancer. Potentially gonadotoxic chemotherapeutic agents have largely contributed to this progress, although this evolution has also led to a significant increase of the incidence of premature ovarian insufficiency. The development of oocyte vitrification as a cryopreservation method [77], has offered new perspectives for the application of IVM in fertility preservation medicine and is now considered as a valuable alternative for ovarian tissue cryopreservation. Since IVM obviates the need for ovarian stimulation, the technique may be especially suitable for patients with estrogen receptor-positive tumors. Chian *et al.* described the first successful live birth following immature oocyte retrieval in a natural menstrual cycle, IVM and vitrification of the IVM oocytes [78], and outcomes of pregnancies achieved with vitrified–warmed oocytes after IVM treatment appear to be favorable [79]. However, larger patient numbers are needed to investigate the risks associated with vitrification of in-vitro matured oocytes. Although in a recent study no difference was found between the fertilization and cleavage rates when oocytes were vitrified at the immature GV stage as opposed the mature MII stage, embryos derived from oocytes that had been vitrified at the immature GV stage are of poorer quality and development to the blastocyst stage appear to be compromised [80].

Finally, the rapid developments in the field of IVM of oocytes should ultimately be adapted to multi-step in vitro follicle culture after ovarian tissue cryopreservation, which, when applied as a fertility preservation strategy, can provide a maximal source of oocytes in female cancer patients and in women with early-onset incipient ovarian failure [81].

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Chapter 36

# Clinical potential of in vitro maturation

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## Introduction

Over the last three decades there has been a significant delay in starting a family in modern Western societies [1]. The most likely reason of this deliberate action is extended life expectancy combined with ever-increasing involvement of women in education and the workforce. However, while men are able to reproduce until much later in life, female fertility declines with increasing age [2, 3]. The current reproductive paradigm suggests women are born with a finite quantity of oocytes and their reproductive potential usually does not last much beyond the end of the fourth decade of life. This is a worrisome fact for many modern women deferring child bearing until their late thirties or even forties. In the year 2007, decreased ovarian reserve was the sole indication for 14% of all reported assisted reproductive technology (ART) cycles in the USA [4]. Moreover, the female partner was older than 35 years of age in 71% of all ART cycles performed in the same year [4].

The natural decline in fertility is markedly accelerated following gonadotoxic therapy for cancer and other non-oncological conditions. Cancer continues to be a major health problem despite advances in its diagnosis and treatment. It is estimated that in 2009 approximately 713 220 women in the USA will be diagnosed with cancer [5]. Survival rates have enjoyed a stable increase over the last decades leading to an increase in the number of cancer survivors every year [5]. Similar advancements occurred in the treatment of childhood cancers, and it is estimated that one in every 250 adults will be a childhood cancer survivor by the year 2010 [6]. Eventually, a growing number of female cancer survivors are faced with the risk of infertility resulting from gonadotoxic oncological treatment. Patients who are exposed to gonadotoxic agents for the treatment of non-oncological diseases, such as systemic lupus erythematosus, those who are undergoing surgery for endometriosis as well as women with genetic disorders such as Turner's syndrome and fragile-X premutation face similar risks, further contributing to the population of women who need fertility preservation procedures [7–10].

Relatively recent advances in the field of reproductive medicine, particularly in cryopreservation methods, have rendered fertility preservation a realistic option for such unfortunate women. With an increased awareness of the options available for fertility preservation, a greater number of women are being offered and are utilizing these technologies. Currently available options for preservation of female fertility are cryopreservation of oocytes or embryos following in vitro fertilization (IVF) or in vitro maturation (IVM) or cryopreservation of ovarian tissue. Although successful transplantation of fresh whole ovary has been reported in a pair of monozygotic twins discordant for ovarian failure and encouraging results have been achieved with frozen ovaries in animal models, cryopreservation of entire ovary has not been successfully performed in humans [11, 12]. Administration of gonadotropin-releasing hormone analogues or inhibitors of apoptosis have been proposed as alternative strategies for patients undergoing chemotherapy; however, effectiveness of these techniques remains to be proved [13]. The most appropriate method for any woman is determined by several factors including the indication for fertility preservation, availability of a male partner and patient preference.

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## IVM and its contribution to fertility preservation

While IVF involves collection and in vitro fertilization of multiple in-vivo matured oocytes collected at the metaphase-II stage (MII), the IVM technique aims to make use of the multiple immature oocytes that already exist in the ovaries of a reproductive-aged woman. Early in the follicular phase, these immature oocytes are harbored in smaller-sized antral follicles, and are arrested at the prophase stage of meiotic division. These oocytes are shown to resume meiosis upon removal from the follicle, have the capacity to complete meiotic division and can be fertilized in vitro. More than 80% of oocytes were reported to resume meiosis independent of the menstrual cycle day and gonadotropin support in IVM medium [14]. In fact, the first pregnancy and live birth from in-vitro matured oocytes in humans was reported in 1983 in the context of a stimulated IVF cycle [15]. However, recovery of mature oocytes following controlled ovarian stimulation (COS) had already become the most popular method, and so IVM did not attract much attention until 1991, when Cha *et al*. reported collecting immature oocytes from women undergoing gynecological surgery, fertilizing these donated oocytes following IVM and transferring the resulting embryos to a recipient woman with premature ovarian failure [16]. The recipient delivered healthy triplet girls. Three years later, Trounson *et al*. from the Monash IVF group reported the collection of immature oocytes from women with polycystic ovary syndrome (PCOS) [17]. The immature oocytes collected were matured in vitro with gonadotropin-enriched medium, then fertilized, and a healthy live birth following transfer of resultant embryos was reported [17]. However, the initial pregnancy rates were low, and it took another 5 years to reach more satisfactory figures exceeding 30% per cycle in appropriately selected patient groups [18, 19].

## Overview of an IVM cycle for fertility preservation

Monitoring starts with a baseline scan performed in the early follicular phase of the menstrual cycle, preferably between days 2 and 5 of a natural menstrual cycle. The number and size of the antral follicles are recorded. The ovaries are examined for any abnormalities. A second scan is performed about a week later when it is anticipated that the largest follicle has reached 10–12 mm. The presence of a dominant follicle does not require cancellation of the treatment cycle because smaller follicles are found to contain viable oocytes, even in the presence of a dominant follicle [20, 21]. Based on our own experience and a favorable trend observed in trials of human chorionic gonadotropin (hCG) priming, the current routine IVM protocol at the McGill Reproductive Centre (MRC) involves hCG administration 10 000 IU im 38 h before oocyte collection. Although the decision regarding timing of the hCG injection, and therefore oocyte retrieval, requires that both the follicle size and endometrial thickness be taken into consideration in a regular IVM cycle, endometrial thickness is ignored in fertility preservation cycles as there will not be an embryo transfer in the same cycle.

Most patients easily tolerate the immature oocyte collection procedure under conscious sedation with intravenous midazolam and fentanyl. Paracervical block is achieved with 1% bupivacaine injection after cleaning the vagina with sterile saline. As the follicles are smaller than the mature follicles aspirated in IVF cycles, a smaller-gauge needle (19–20 G) with a shorter bevel is preferred. The aspiration pressure is set at 75–80 mmHg, approximately half the conventional IVF aspiration pressure, in order to minimize the risk of oocyte denudation during aspiration. The follicles are often widespread throughout the ovarian stroma and aspirating all of them with a single puncture is generally impossible. Moreover, the fine-bore needle may be blocked frequently with bloodstained aspirate and ovarian stroma. Therefore, multiple punctures are often needed and flushing the needle lumen with heparinized saline between punctures is required. Sometimes external abdominal pressure may be required to fix the mobile ovaries during collection. Patients with difficult-to-reach ovaries or poor pain control may do better under limited general anesthesia with propofol.

## Obstetric outcome of IVM

In vitro maturation has become an effective treatment option for many infertile women resulting in the birth of over 2000 healthy infants [18, 19, 22, 23]. We have found the mean birth weights of infants conceived with IVM, IVF or intracytoplasmic sperm injection (ICSI) to be similar, but lower than those of spontaneous conceptions [24]. The proportion of low-birth-weight and very low-birth-weight infants was

similar across ART children. The proportion of infants with an Apgar score ≤6 at 1 and 5 min and the incidence of acidosis were all similar among IVM, IVF, ICSI or spontaneous-conception deliveries [24]. Compared with spontaneous conceptions, the observed odds ratios (ORs) for any congenital abnormality were 1.42 (95% confidence interval [CI] 0.52–3.91) for IVM, 1.21 (95% CI 0.63–2.32) for IVF and 1.69 (95% CI 0.88–3.26) for ICSI, respectively. None of these were statistically significant [24]. This provides indirect evidence that the reported high congenital abnormality rate with ICSI is due to poor sperm per se, because ICSI with normal sperm did not increase the odds of congenital abnormality to the same extent in IVM cycles. In a retrospective study, the chromosomal constitution and mental development of 21 children born after IVM were compared with 21 spontaneously conceived children. All of the IVM children were found to have normal karyotype and mean developmental index score similar to controls in this study [25]. Another study of 46 IVM babies born to 40 women in Finland reported similar findings [26]. The physical growth of IVM children seems to be similar to that of spontaneously conceived children [25, 26].

Young women with high antral follicle counts seem to have the highest pregnancy rates with IVM [27]. In vitro maturation has become an established treatment option for women with polycystic ovaries (PCO) or PCOS who need ART. However, the clinical application of IVM technology is not limited to these women alone, and can be extended to benefit other patient populations. In vitro maturation, especially when combined with oocyte vitrification, provides unique opportunities for women who wish to preserve their reproductive potential.

## IVM for fertility preservation

At present, embryo cryopreservation following IVF is the only method endorsed by the American Society of Clinical Oncology (ASCO) and the American Society for Reproductive Medicine (ASRM), while the other methods are still considered experimental [28, 29]. In fact, it is the "tried and true method" as the successful cryopreservation of surplus embryos after IVF and resultant pregnancy following frozen–thawed embryo transfer (FET) was first reported in 1983, and the first child after embryo freezing was born in 1984 [30, 31]. It is estimated that almost one quarter of the children born after ART are born following cryopreservation

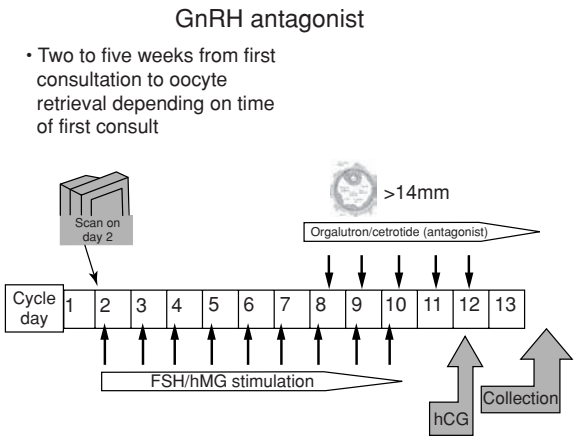


**Figure 36.1** Overview of the gonadotropin-releasing hormone (GnRH) antagonist protocol. FSH, follicle stimulating hormone; hMG, human menopausal gonadotropin. From a presentation on fertility preservation by Dr. S. L. Tan, with permission.

of mostly cleavage-stage embryos and, less commonly, blastocysts and oocytes [32]. However, several points raise concern about IVF–embryo freezing in cancer patients. These are: (1) a possible delay of 2–5 weeks in treatment of the primary disease due to ovarian stimulation depending on the timing of the first consultation with the reproductive endocrinologist in relation to onset of the next menstrual cycle; (2) exposure to supraphysiological estrogen levels induced by ovarian stimulation; (3) the requirement for a male partner or willingness to use donor sperm for embryo production; and (4) legal, ethical, religious issues related to cryopreservation of embryos in general. These issues and alternatives provided by IVM are discussed below.

## Problems with ovarian stimulation in cancer patients

The time required for completion of the fertility preservation procedure, which starts with the initial reproductive medicine consultation and technically ends with oocyte collection, depends on the conditions of any particular clinic. Ovarian stimulation takes between 2 and 5 weeks, depending on the stimulation protocol employed and the timing of the following menstrual cycle of the patient (Figure 36.1). This is a matter of concern for both the patients and treating physicians. The effect of such a delay in treatment obviously depends on the underlying disease and must be evaluated on a case-by-case basis together with the treating oncology team.

Serum estradiol (E2) levels are increased during ovarian stimulation for IVF and can reach levels 20 times higher than those of a natural cycle [33]. Breast cancer remains the most common cancer in females, representing 27% of all female cancers. Between 2002 and 2006, 12.4% of all breast cancer cases were diagnosed in women younger than 45 years, who are in their reproductive period [34]. The risk of breast cancer is consistently found to be associated with persistently elevated blood estrogen levels [35]. Although the effect of a temporary increase in serum E2 levels on the risk of recurrence of breast cancer is controversial, this remains another fact causing concern among both physicians and patients. Such concerns are not limited to women with estrogen-receptor positive breast cancer, because recent findings also suggest the presence of an indirect mitogenic effect of estrogen on hormone-receptor negative breast cancer [36]. Increased E2 levels can be relevant for patients undergoing fertility preservation treatment due to other oncological or non-oncological diseases considered to be estrogen sensitive, such as desmoid tumors, systemic lupus erythematosus or severe endometriosis. Special stimulation protocols involving aromatase inhibitors in order to limit the rise in estradiol levels have been developed for such patients [37]. Despite encouraging results with regard to disease-free survival and recurrence rates being reported by the authors, it is interesting to note that 63.3% of breast cancer patients referred for reproductive endocrinology and infertility (REI) consultation at the same center declined ovarian stimulation and IVF due to concerns about delay of chemotherapy, effect of ovarian stimulation on cancer or costs associated with treatment [37].

## Advantages of IVM

In vitro maturation avoids these two important concerns which prevent a substantial amount of patients at risk of infertility using them. Immature oocyte retrieval in an unstimulated menstrual cycle or from ovarian tissue biopsies followed by IVM and oocyte or embryo cryopreservation provides a novel fertility preservation strategy [38, 39]. Avoiding ovarian stimulation provides several important advantages for cancer patients. Compared to 2–5 weeks required for a stimulated IVF cycle, immature oocyte retrieval can be done within 2–10 days, depending on the patient's menstrual status [39]. Immature oocytes can be collected even in the luteal phase. We reported three women without male partners seeking fertility preservation prior to chemotherapy who presented for the first time in the luteal phase of their menstrual cycle and were to undergo gonadotoxic treatment immediately. Five to seven immature oocytes were recovered by luteal-phase oocyte retrieval from these women. Three to five MII oocytes were vitrified following IVM. Two of the three women later underwent one and two more collections, respectively, in the follicular phase of the next cycle(s) and additional immature oocytes were vitrified following IVM [21]. Moreover, immature oocyte collection in the luteal phase provides a rescue option for a patient who experiences a premature LH surge during ovarian stimulation [40]. Although cancelling the treatment cycle can be an option for the regular patient, cancer patients undergoing fertility preserving treatment usually don't have time for a new treatment cycle and require immediate solutions. We were able to collect four immature oocytes in a breast cancer patient who had a premature LH surge during an ovarian stimulation cycle started for fertility preservation in New York. She had experienced an abrupt increase in serum LH level on the 7th day of stimulation. Progesterone level reached 8.38 ng/ml on the 10th day of stimulation. On the same day, the leading follicle size was 20 mm, and there was no sonographic finding suggesting ovulation: 10 000 IU hCG was administered and collection scheduled 35 h later. At the time of collection there were approximately 10 follicles sized $\geq$10 mm, and 4 of them had the appearance of corpus lutei accompanied by free fluid around the ovaries. Nevertheless all follicles were aspirated, resulting in collection of 4 immature oocytes at the germinal vesicle (GV) stage from follicles $<$10 mm in average diamater. Two GV oocytes were matured in vitro and fertilized successfully, resulting in vitrification of two embryos [40].

In addition to avoiding potential delay in treatment and rise in estradiol levels, IVM eliminates the risk of ovarian hyperstimulation syndrome (OHSS). This syndrome is a major complication of COS, characterized with increased vascular permeability [41]. Clinically, it manifests as ascites, pleural effusion, hypovolemia, hemoconcentration and hypercoagulability. Severe cases can be complicated by thromboembolism or even death. When superimposed on cancer, OHSS can have serious consequences on the health of these patients and can cause further delay in pending oncological treatment.

## Collection of immature oocytes from ovarian tissue specimens for cryopreservation

Immature oocytes can also be harvested from ovarian biopsy specimens and fertilized or vitrified following IVM [38]. This combination of ovarian tissue cryobanking and IVM represents a new strategy for fertility preservation [9]. We retrieved 11 immature oocytes from a wedge resection specimen in a 16-year-old patient with mosaic Turner's syndrome. Eight of these oocytes were vitrified following IVM [9]. In 4 women with cancer (2 Hodgkin's lymphoma, 1 breast cancer and 1 rectal cancer), we harvested 11 immature oocytes from wedge biopsy specimens collected for ovarian tissue cryopreservation. Patient age ranged between 18 and 38 years. In two patients, surgery was performed in early follicular phase and another two women underwent surgery in the luteal phase of the menstrual cycle. The median number of immature oocytes collected was three. Eight of the eleven immature oocytes reached MII stage following IVM and were vitrified [38].

## IVM: oocyte vitrification

Requirement for a male partner and ethical/legal/religious issues associated with embryo cryopreservation are other aspects that require attention when IVF-embryo cryopreservation is considered as a fertility preservation measure. Although infertility affects a couple in general, cancer affects the individual. Fertility preservation should aim to preserve not only the individual's germ line, but also her autonomy for her own reproductive potential. For single women, generating then freezing embryos conceived with donor sperm is obviously not the same as cryopreserving unfertilized oocytes for possible use with a future partner. On the other hand, while donor sperm is not required for a woman who has a partner, freezing embryos means sharing the control over the embryos with him. Unfortunately, some couples split up in the face of cancer. In the case of separation, the former male partner also has rights over the embryos, with all possible legal and ethical implications. The ex-male partner may disagree to using the embryos and to conceive a child, as in the case of Evans versus Johnson [42]. In this famous case, Ms. Evans had an IVF cycle before undergoing surgery for ovarian cancer, and her oocytes were fertilized using sperm from her fiancée at that time. The couple split up prior to completion of her treatment. The ex-fiancée withdrew his consent and asked for the embryos to be destroyed. Ms. Evans started a lawsuit and following the British High Court ruling against her wish to continue with embryo transfer, the European Council of Human Rights also ruled against her. The embryos were eventually destroyed. If there is any doubt, it seems better to freeze oocytes since oocytes belong to the woman, while embryos belong to the couple. We believe that in contrast to embryo cryopreservation, oocyte cryopreservation provides the most effective means of ensuring the reproductive autonomy of the patient. Another advantage of oocyte cryopreservation is that it avoids the ethical and religious quandaries associated with the storage and disposal of embryos.

Oocyte cryopreservation is considered an investigational procedure in the ASCO and ASRM reports as well as in a working party report of the Royal Colleges of Physicians, Radiologists and Obstetricians and Gynaecologists on the management of cancer patients undergoing gonadotoxic treatment [28, 29, 43, 44]. The major motivation behind this opinion was that a relatively better clinical outcome was being reported with embryo than with oocyte cryopreservation.

However, these opinions by ASRM, ASCO and the Royal Colleges are based on data published prior to 2005, 2006 and 2007, respectively. A meta-analysis of the efficiency of oocyte cryopreservation published in 2006 reported live birth rates of 1.9 and 2.0% per oocyte thawed after slow freezing and vitrification, respectively [45]. The vast majority of data on oocyte cryopreservation were from experience with slow freezing at that time, and a substantial amount of relevant data favoring vitrification has subsequently been published [46–51]. In a clinical trial at the McGill Reproductive Centre, Montreal, Canada involving 38 infertile women who underwent oocyte collection in a gonadotropin-stimulated cycle, oocyte vitrification using the McGill Cryoleaf™ resulted in a mean survival rate of 81% post-thawing, a 76% fertilization rate, a clinical pregnancy rate per cycle of 45%, a live birth rate of 40% and 22 healthy babies [52]. In a review of 165 pregnancies and 200 infants conceived following oocyte vitrification, the birth weight and the incidence of congenital anomalies (2.5%) were comparable to those following spontaneous conception or IVF treatment [53]. A more recent review corroborates our observations [54].

**Table 36.1** The number of patients to date who underwent different fertility preservation procedures at the McGill Reproductive Centre, Montreal, Canada

| Malignancy | IVM/EV | IVF/EV | IVM/OV | IVF/OV |
|---|---|---|---|---|
| Hematological | 7 | 10 | 15 | 15 |
| Breast | 31 | 1 | 36 | 3 |
| Gynecological | 1 | 2 | 4 | 7 |
| Brain | 2 | 3 | 5 | 5 |
| Sarcoma | 1 | 1 | 3 | 6 |
| Gastrointestinal tract | 1 | 1 | 1 | 3 |
| Melanoma | 0 | 0 | 0 | 1 |
| Autoimmune diseases | 1 | 1 | 4 | 3 |
| Desmoid tumor | 1 | 1 | 2 | 1 |
| **Total** | **45** | **20** | **70** | **44** |

EV, embryo vitrification; IVF, in vitro fertilization; IVM, in vitro maturation; OV, oocyte vitrification.

Similar to in-vivo matured oocytes, in-vitro matured oocytes can be successfully cryopreserved with vitrification, further expanding the choices for women who wish to preserve their fertility potential. Although smaller sized immature oocytes without the meiotic spindle can be anticipated to better survive cryopreservation, laboratory and clinical outcomes of cryopreservation of oocytes at the GV stage by slow freezing or vitrification have not been as good as those achieved with mature oocytes [46]. Despite similar survival rates for GV and MII ocytes after vitrification (85.4 versus 86.1%, respectively), the maturation rate of vitrified–warmed GV oocytes is significantly lower than that of fresh GV oocytes (50.8 versus 70.4%, respectively) [46]. However, the fertilization rate and embryo development rate are similar for vitrified or fresh GV oocytes once they reach MII stage. Given these facts, it seems cryopreservation of immature oocytes after they have matured to MII stage seems a better strategy. In a pilot study at the McGill Reproductive Centre on IVM oocyte vitrification, a live birth rate of 20% per cycle was achieved, including the world's first four live births from vitrified IVM oocytes [55]. Compared to in-vivo matured oocytes collected following ovarian stimulation, IVM oocytes had a significantly lower survival (81.4 versus 67.5%) and fertilization (75.6 versus 64.2%) rates following vitrification–warming. Despite a trend towards lower implantation (19.1 versus 9.6%), clinical pregnancy (44.7 versus 20.0%) and live birth (39.5 versus 20%) rates with IVM oocyte vitrification, none of the differences were statistically significant [55]. The clinical outcome of IVM oocyte vitrification is regarded satisfactory for patients whose conditions preclude ovarian stimulation for any reason. To date, the McGill Reproductive Centre has provided fertility preservation to 180 patients with breast, hematological, brain, soft tissue, colorectal and gynecological cancers: more than 100 of these women have oocytes or embryos cryopreserved following IVM (Table 36.1). A suggested algorithm for cancer patients is presented in Figure 36.2 [56].

## IVM for social fertility preservation

Both the ASRM and European Society of Human Reproduction and Embryology (ESHRE) recommendations are against ovarian tissue cryopreservation in healthy women who are not faced with an "immediate threat to fertility" [43, 57]. Similar to ovarian tissue freezing, oocyte cryopreservation is also regarded an experimental procedure, and both societies find it early to "recommend" or "encourage" oocyte freezing without a medical indication [43, 57]. The risk to benefit ratio of the procedure is one of the major concerns. In vitro maturation provides an unique opportunity of oocyte freezing without ovarian stimulation for women who wish to do delay child bearing for social reasons. In-vitro maturation oocyte vitrification avoids risk of OHSS and the inconvenience of daily gonadotropin injections for these healthy women. The direct and indirect costs of treatment, i.e. cost of gonadotropins, loss of working days for monitoring scans etc., are less with IVM. Based on this, IVM can be

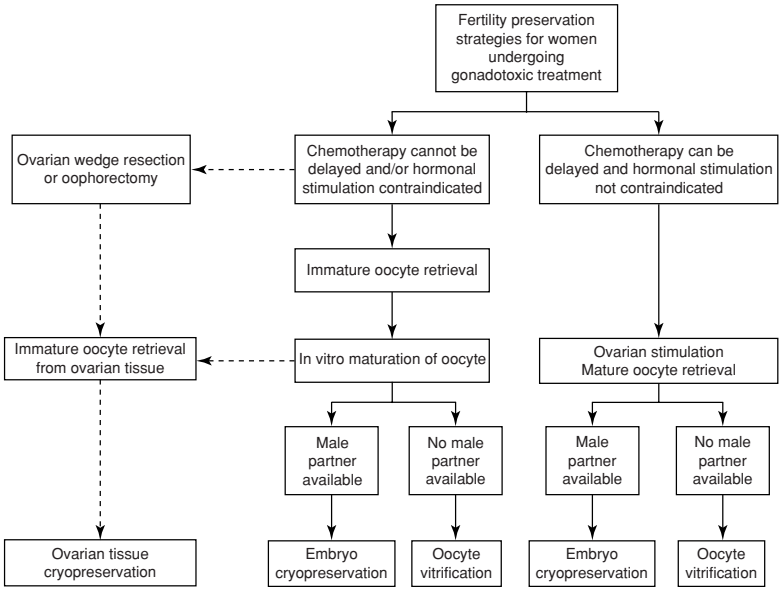


**Figure 36.2** Suggested algorithm for fertility preservation. Reproduced from Chian *et al.* [56]. with permission from Elsevier Science, Inc. © 2009 American Society for Reproductive Medicine.

considered a simpler and safer procedure and regarded more acceptable.

In conclusion, IVM combined with embryo or oocyte vitrification provides previously unavailable options for some patients and improves the services provided by a fertility preservation program. Primary care physicians and oncologists need to be made aware of the available fertility preservation options in order to allow early discussion with their patients followed by referral, if desired, to an ART center that offers a full range of fertility preservation options.

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Chapter 37

# From pluripotent stem cells to germ cells

Rosita Bergström and Outi Hovatta

## Introduction

Infertility problems represent the biggest health issue among people aged 20–45 years, and a major concern is that the magnitude of this problem may be increasing, as many couples wait until later in life trying to build a family. It is speculated that the latter may be part of the reason why the 2005 National Survey of Family Growth report found a 20% increase in American couples experiencing infertility problems between 1995 and 2002 [1].

The number and quality of oocytes is reduced with age, but it may also decrease as a consequence of a disease or treatment [2, 3]. In males, spermatogenesis normally continues during adult life, but the number and quality of fertile sperm cells is, as for females, affected by various factors, often following disease [4, 5]. Yet very little is known in this area of research, principally due to lack of models for human germ cell development. Selected differentiation of pluripotent stem cells to germ cells opens up a door to not only learning more about germ cell development in general, but also as a tool to study specific causes of infertility. Differentiation of gametes from pluripotent human stem cells may constitute a therapeutic option for infertile couples in the future.

## Stem cells

Stem cells are undifferentiated cells characterized by their ability both to renew themselves and to give rise to various types of specialized cells. Adult stem cells are multipotent, i.e. they have the ability to differentiate to a limited number of cell fates, and can be obtained from various tissues, including blood [6], bone marrow [7], fat [8], skin [9] and testis [10].

## Embryonic stem cells

Human embryonic stem cells (hESCs) are pluripotent, meaning that they have the ability to give rise to a wide range of cells belonging to all three germ layers (ectoderm, endoderm and mesoderm) as well as into germline lineage [11]. These cells can be derived from the inner cell mass of blastocysts [12], cleavage stage embryos or single blastomers [13–15] Extraembryonic tissues, such as the amniotic fluid, umbilical cord blood and the placenta can give rise to multipotent stem cells. Such multipotent cells have more limited differentiation capacity than pluripotent embryonic stem cells (ESCs) and induced pluripotent cells. Regarding reproductive biology and medicine, the most interesting cells are two types of pluripotent stem cells (i.e. human embryonic and induced) and testicular and ovarian stem cells.

## Stem cells from testis and ovary

Pluripotent stem cells have been identified in the seminiferous tubules of the testis in rodents and also, lately, in humans [16, 17]. In the future, these cells may be important tools for studying human spermatogenesis and for obtaining mature sperm in vitro after fertility preservation in pre-pubertal boys. It would be less risky than transplantation of cells, particularly in hematological malignancies. So far, spermatogenesis in vitro has not been completed, but there are promising results in animal experiments, particularly in three-dimensional cultures [18].

Stem cells in the ovary have been discussed intensively during the recent years and, for the time being, this remains a controversial issue. Presently there is no convincing evidence of oocyte-forming stem cells being found in the postnatal human ovary.

*Principles and Practice of Fertility Preservation*, eds. Jacques Donnez and S. Samuel Kim.
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## Induced pluripotent stem cells

In 2006 there was a major breakthrough in the field of stem cell research. Yamanaka's group were, for the first time, able to reprogram somatic, specialized cells into pluripotent stem cells. The cells in questions were mouse fibroblasts, that upon transfection with retroviruses carrying the four key pluripotency genes Oct-3/4, Sox-2, c-Myc and Klf4, spontaneously dedifferentiated into stem cell-like cells, so called "induced pluripotent stem cells" (iPSCs) [19]. By altering the expression of these genes alone, the fibroblasts spontaneously dedifferentiated into iPSCs. In the sense of morphology, proliferation and teratoma formation these cells were similar to hESCs, but there were severe epigenetic errors such as abnormal DNA methylation patterns and faulty gene expressions. Furthermore, the iPSCs failed to produce viable chimeras when injected into developing embryos.

Successful reprogramming of mouse fibroblasts into iPSC and the production of viable chimeras were obtained in 2007, by replacing $Fbx15^{+}$ with Nanog for isolation of cells by antibiotic selection [20]. However, 20% of the chimeric mice developed cancer, likely due to the overexpression of the oncogene c-Myc, used to induce the dedifferentiation and/or epigenetic errors. At the end of 2007, Yamanaka's team managed to repeat their results in adult human cells using the same four genes as in previous studies performed in mice [21]. The concerns using c-Myc remained, but nevertheless this was a big step forward in the field of reproductive medicine in humans.

Also at the end of 2007, Thomson and colleagues managed to create human induced pluripotent stem cells (hiPSCs) avoiding the use of oncogenes. Instead they used a lentiviral system based on the expression of OCT4, SOX2, NANOG and LIN28 [22]. LIN28 is a marker of undifferentiated hESC and is associated with several epigenetic features, such as micro-RNA and the regulation of the imprinted gene Igf2, but the exact mechanisms behind this remain to be investigated.

The technical limitations obtaining iPSCs has been a concern for its applications in humans. Using a virus with random insertions in the human DNA is a dangerous approach as it may cause mutations in the DNA code and/or cause epigenetic errors (epimutations). Following these initial, but for therapeutic applications insufficient, breakthroughs, inducible lentiviral systems have been developed to generate iPSCs [23–25]. Using this strategy, however, the drawback of permanent integration in the host genome remains. Lately non-integrating approaches, like adenoviral delivery [26], transient transfection [27] and direct delivery of reprogramming factor proteins [28] have been successfully used in the reprogramming of mouse and human cells. The efficiency using these methods has to be improved before they can be applied for clinical purposes, but this gives great hope for the future.

## Epigenetic regulation

The DNA code is more or less the same in all the cells of the human body. What differs is the way this code is utilized or, in other words, which combinations of genes that are active in a specific cell at a given time. The secret behind this is the epigenetics, the heritable changes in gene function that occur without a change in the sequence of the DNA [29, 30]. This includes a wide range of mechanisms, such as DNA methylation, histone modifications, genomic imprinting and higher order chromatin conformation [29]. The overall epigenetic state of a cell is referred to as the "epigenome." The creation of the diverse cell types from pluripotent stem cells depends ultimately on the establishment and maintenance of specific patterns of gene expression, a process under careful control of epigenetic features. This information can be reversed and changed, but also passed on through cell divisions and generations, such as an "epigenetic memory."

There is an extensive cross talk between different types of epigenetic regulation that carefully adjust the level of gene expression at any given moment. Some adjust rapidly upon a stimulus and others remain steady through cell divisions, distinguishing different cell types. This fine-tuned control of gene expression is the key factor behind stemness and differentiation, and it is an absolute necessity that we learn to understand these features in order to continue making progress in the field of stem cell research and differentiation. What is the difference between various types of cells? How many different cell types are there in the human body? This is a key question if we are to create one kind of cell from another. However, first we need to define "cell type." Depending on who answers, the answer will probably range from "a few hundred" to "a few million." The epigeneticist would most likely state that there are as many types of cells in the human body as there are number of cells in total, because no cell is epigenetically the same as another. The approximately 2 m of DNA is modified and folded in an unique way

in each and every cell. However, there are similarities between cells with distinct functions. Germ cells are, for example, epigenetically similar – but not identical – to one another.

The three-dimensional positioning of the chromosomes in relation to each other, or chromosome territories, is one of the features that differ between groups of cells. That is, the locations of the chromosomes, their neighbors and the interactions/co-regulations vary between different cells. This is important in the field of germ cell differentiation and development, since the degree of difference in the epigenome between the starting material (the cells to be reprogrammed) and end product (germ cells) will vary depending on which starting material is utilized and the epigenetic statues of these cells.

In line with this, it has been reported that using the same protocol, differences in time and success rate for reprogramming somatic cells to iPSCs varies with the choice of starting material [31]. This is not surprising, it makes sense that it is less of an effort to make one cell from another that is more alike the end product. Due to this, it might be a good strategy using explicit protocols for deriving iPSCs from various starting populations, and to consider that lines that are epigenetically more different from pluripotent cells may need longer time to adjust their epigenome in to a pluripotent state. This strategy may very well also apply for differentiating stem cells of various kinds to germ cells. One may not experience that the differentiation process will take the same amount of time, depending on the potential differences in the epigenomes.

A major concern in iPSCs derivation (and all other kinds of reprogramming, such as any type of differentiation) is *incomplete* reprogramming [19, 32, 33]. Cells that are stuck somewhere in between two states can be difficult to interpret. The expression of a handful of pluripotency markers is not enough to reprogram the cell on their own, and it is expected that DNA methylation and histone modifications play important roles [34]. For instance, the promoter regions of many pluripotency associated genes are heavily methylated in somatic cells, repressing the expression of the corresponding transcripts. For completion of reprogramming these promoters need to be hypomethylated, as they are in ESCs [35], a process that takes several cell cycles to complete. The use of demethyation-promoting agents, such as 5-azacytidine during iPSCs derivation, has proven to effectively support this process [32].

The patterns of histone modifications are other key features for complete reprogramming. In differentiated cells, histones H3 and H4 are hypoacetylated in promoter areas of pluripotency genes, and their expressions are silenced. In order for proper pluripotency-specific gene expression, i.e. complete reprogramming, hyperacetylation needs to occur. Use of the histone deacetylation inhibitor valproic acid has proven to efficiently enhance the generation of iPSCs, probably due to the enhancement of acetylation [36, 37].

The purpose of iPSCs is that they should mimic the nature of hESCs. Therefore, iPSCs should be cultured in the same way as hESCs. If they were not to be cultured in the same manner, we would have completely failed making the kinds of cells we attempted. However, there are concerns with iPSCs that we must take under consideration during cell culture. First of all, there is a risk that these cells are not fully reprogrammed and will differentiate back to the kind of cells they were before reprogramming; therefore, there is a concern that these cells will be more difficult to differentiate towards other lineages, such as germ cells. This is possible if the epigenetic memory is not sufficiently reprogrammed. Overexpression of a limited number of factors alone will not keep cells in a pluripotent state if the epigenome is set at a differentiated state.

Cell culturing is also challenging due to the problem with culture adaptation [38]. The iPSCs may even be more unstable than ESCs, as the epigenome can be out of equilibrium and the cells may "fumble in the dark," awaiting signals or stimuli, in order to rapidly differentiate in to a more stable state. Recently, however, it has become clear that alternative culturing conditions, such as hypoxia, may improve the efficiency of iPSCs [39].

There is no doubt that many epigenetic phenomena take place during reprogramming, and by learning more about how these function there is great room for improvement for controlled reprogramming of cells. Perhaps epigenetic features can be used as a tool to investigate the status of reprogramming. And perhaps epigenetics can elucidate some of the many cases of unexplained of infertility.

## Epigenetic regulation of germ cell development

The development of germ cells is a highly ordered process that begins during fetal growth and is completed

in the adult. Epigenetic modifications are sequentially established and erased in the germ cell lineage. This epigenetic reprogramming is essential for the acquisition of totipotency and the epigenetic marks called imprints that distinguish the parental origin of about 80 genes in humans [40]. The imprinted marks are erased in primordial germ cells (PGCs) and then reset in a parent-of-origin specific manner, such that they are in place at the time of fertilization.

In the 1980s, it was demonstrated how crucial imprinting is for normal development. Two female (biparental gynogenones) or two male (biparental androgenones) pronuclei were used with the attempting to construct diploid mouse embryos [41, 42]. The ability of these embryos to develop to term was compared with control nuclear-transplant embryos in which either the male or female pronucleus was replaced with an isoparental pronucleus from another embryo. The results revealed that, in contrast to the controls, neither diploid biparental gynogenetic or androgenetic embryos completed normal embryogenesis. Interestingly, the outcome of the experiment also showed to be different for the two setups. Whereas the gynogenetic embryos showed relatively normal embryonic development but poor placenta development, the androgenetic embryos showed poor embryonic development but normal placental development. This experiment points out how important parental imprinting is for certain stages of development, and also explains why parthenogenesis does not exist in humans.

Twenty years later, experimental manipulation of the imprinting locus *H19/Igf2*, forcing unequal expression of this loci from the two pronuclei (i.e. forcing an artificial imprinted state), allowed the creation of rare individual mice with two maternal sets of chromosomes [43]. Interestingly, several other imprinting centers were affected due to manipulation of this region alone. This observation is perhaps not that surprising, as the imprinted locus in question has been pointed out as a potential key point for higher order chromatin conformation [44, 45] and is likely to affect the regulation of several other loci. Whether this is a unique feature of the *H19/Igf2* locus or is true for all imprinted regions is still to be determined.

The global degree of methylation is about the same in the genomes of mature eggs and sperm as it is in somatic cells, while the genome in stem cells is hypomethylated. The specific pattern of methylation is, however, unique in various cell types. Following the genome-wide demethylation that occurs in both male and female germ cells in the early development of the PGCs, the cells enter mitotic (male) and meiotic (female) arrest, respectively [46, 47]. Interestingly, the subsequent remethylatin occurs much earlier in the male germ line, at the prospermatogonia stage, than in the female line where remethylation takes place after birth during the growth of the oocytes.

## Germ cells from ESC

Due to their plasticity and potentially unlimited capacity for self-renewal, hESCs have been popular candidates for various cell therapies, including regenerative medicine and tissue replacement. Lately there has been a lot of focus on the potential use for hESCs in reproductive medicine [11, 48–50]. They offer excellent models for studying the regulation of human oogenesis and spermatogenesis, which are not easily accessible for research.

Establishing functional gametes, from pluripotent cells, particularly from patient-specific induced pluripotent cells, would offer totally new options for individuals who lose their own gametogenesis in connection with cancer therapies.

## Oocytes from mouse ESCs

Differentiation of mouse embryonic stem cells (mESCs) to oocytes was first described by Hübner *et al.* [51]. Oocytes were differentiated in adherent cultures from a mouse stem cell line which expressed green fluorescent protein (GFP) under influence of the Oct4 promoter. Populations were enriched by selecting VASA and c-kit expressing cells. Starting from day 12, increasing clusters of cells expressing early and more mature oocyte markers (such as GDF-9) were detected. With time, the meiotic marker synaptonemal comples protein 3 (SYCP3) was activated, which also had several morphological features on oocytes. More mature meiosis markers were not demonstrated. In long-term cultures, embryo-like structures and blastocyst-like formation were seen. The oocytes were not fertilized and must have undergone parthenogenetic activation.

Oocyte formation from mESCs was carefully studied by Novak *et al.* [52]. Embryoid bodies were formed, and the development of oocytes was enhanced in co-culture with a bone morphogenetic protein 4 (BMP-4)-producing cell line. A panel of meiosis specific markers was systematically studied. Out of these,

SYCP3 alone was detected in the germ cell-like cells, and the nuclear distribution of SYPCP3 was highly abnormal. The germ cell-like cells did not contain synapsed homogous chromosomes, but instead displayed a chromosomal organization normally seen in somatic cells. The germ cell-like cells differenriated from mESCs failed to undergo normal meiosis.

The obstacle of getting mature oocytes from mESCs in vitro was overcome by Nicholas *et al.* [53], who first established fundamental parameters of oocyte development during ESC differentiation. They created a timeline of definitive germ cell differentiation from ESCs in vitro that initially parallels with endogenous oocyte development in vivo by single-cell expression profiling and analysis of functional milestones, including responsiveness to defined maturation media, shared genetic requirement of Dazl and entry into meiosis. Because ESC-derived oocyte maturation ultimately failed in vitro, they transplanted ESC-derived oocytes into an ovarian niche to direct their functional maturation. By including this step, mature oocytes, which could be fully characterized, were obtained and they could identify the regulational mechanisms controlling oocyte development.

## Male germ cells from mESCs

In 2003, Toyooka *et al.* [54] established functional male germ cells from mESCs. They used knock-in ESCs, in which GFP or lacZ was expressed from the endogenous mouse vasa homolog, MVH, which is specifically expressed in differentiating germ cells. Using these cells they visualized germ cell production during in vitro differentiation. The appearance of MVH-positive germ cells depended on embryoid body formation and was greatly enhanced by the inductive effects of bone morphogenic protein (BMP)4-producing cells. After transplantation to seminiferous tubules these cells reconstituted testicular tubules, demonstrating that ESCs can produce functional germ cells in vitro.

Primordial germ cells were identified from embryoid bodies by Geijsen *et al.* [55] and continuously growing lines of embryonic germ cells were derived. Embryonic germ cells showed erasure of the methylation markers (imprints) of the Igf2r and H19 genes, a property characteristic of the germ lineage. Embryoid bodies supported maturation of the PGCs into haploid male gametes, which, when injected into oocytes, restored the somatic diploid chromosome complement and developed into blastocysts.

## Germ cells from hESCs

Germ cells were first differentiated from hESCs by Clark *et al.* [49]. The hESCs were differentiated as embryoid bodies, and a shift in expression from RNA and protein markers of immature germ cells to those indicative of mature germ cells, including expression of VASA, BOL, SCP1, SCP3, GDF-9 and TEKT1, was detected, with all markers specific to gonocytes.

The next step taken by the same team [48] was to use stimulation of differentiation with growth factors, BMP-4, BMP-7 and BMP-8b. The cells which expressed the germ-cell specific marker VASA, a factor first identified in 2000 as specific for PGCs [56], were enhanced in culture by BMP stimulation. These cells also expressed SYCP3 as a meiotic marker.

In elegant experiments, the same team then differentiated hESCs in adherent culture instead of embryoid bodies [11]. BMP stimulation was used as before. Silencing and over-expression of the germ-cell specific RNA-binding proteins DAZL, DAZ and BOULE significantly enhanced postmeiotic germ cells in culture. First PGC markers (DAZ1, PRMI1, Stella, VASA) appeared, followed by meiotic markers gamma-H2AX and SYCP3. Typical epigenetic reprogramming, hypomethylation of H19, was detected. Haploid chromosomes as revealed by fluorescence *in situ* hybridization (FISH) were identified in the postmeiotic cells.

Recently, PGCs and, later, postmeiotic spermatids were obtained from hESCs [57]. Spontaneous differentiation in embryoid bodies was carried out, and quantitative real-time polymerase chain reaction (RT-PCR) and immunolocalization was used to identify stepwise the formation of PGCs and then spermatogonial lineage cells, ending in sperm-specifc proteins, such as protamine I and protamine 1.07. Gene expression profiles characteristic of oocyte development and follicle-like structures was seen, but no committed oocytes with zona pellucida. Steroid secretion could be measured in these cultures.

The present knowledge regarding gametogenesis in vitro starting from ESCs already gives us new insight about regulation of human gametogenesis. It may be possible to use this knowledge in identifying causes of infertility and mechanisms of action of toxic factors; for example, those used in chemotherapy. Getting

functional gametes for research and for treatment is a more distant goal.

## Germ cell differentiation from induced pluripotent cells

Renee Reijo Pera and co-workers were first managing to differentiate germ cells from hESCs in vitro [49]. Recently, this method has been improved and also applied to iPSCs [58]. By co-culture of the differentiating cells with human fetal gonadal cells, Park *et al.* significantly improved the efficiency of generating in-vitro derived PGCs, using BMP stimulation, from hESCs, and managed to repeat the experiments using hiPSCs, originating from human skin fibroblasts [58]. Interestingly, the authors did not detect any major differences in the efficiency of germ cell differentiation between using hESCs or hiPSCs. After 7 days of differentiation the in-vitro derived PGCs were transcriptionally distinct from the somatic cells (showing expression of genes associated with pluripotency and germ cell development). In addition, by use of bisulfite sequencing, signs of initiation of imprinting erasure was detected in the population originating from hESCs but not from hiPSCs after this time period. In conclusion, this suggests that iPSC derived from fetal cells may differentiate to PGCs, but there is so far no evidence if this is also true for iPSCs derived from adult somatic cells. Neither is there currently any available evidence that iPS-derived PGCs can enter or go through meiosis, a diagnostic property of germ cells.

## Potential applications and future prospects

The underlying genetic and epigenetic mechanisms behind germ cell differentiation are poorly understood in humans due to a lack of models. Extensive, and essential, epigenetic modifications have however been observed to occur in *Caenorhabditis elegans* and *Drosophila*, among other model organisms, during germ cell development [29]. Technical progress in the field of molecular biology and epigenetics has lead to a rapid development of the knowledge in this area during the last decade. Nevertheless, there are still major obstacles that need to be solved, not the least in the area of germ cell development and fertility. Hopefully the combination of iPSCs derivation and in-vitro germ cell differentiation will shed some light over this process. The nature of the epigenome is that it is always changing (as responses to various stimulus, from within the cell or the surrounding), and this dynamic feature is technically challenging to investigate. In particular, there are very few single cell analyses available, where the epigenome of one induvidual cell could be measured.

The use of stem cells has provided a research tool to study development and disease in details never before possible. But even though treatment using stem cells is giving great hope towards the cure of various human disorders, such as leukemia, brain damage, cancer and infertility, there are many fundamental questions that still need to be answered, not least at an epigenetic level. How do we ensure that cells differentiated in vitro have the same phenotype as corresponding cells differentiated in vivo? Which are the fundamental genetic and epigenetic steps during differentiation and development?

In the future there may be potential for using gametes differentiating from hESCs or hiPSCs as a therapy for infertile couples. The advantage of using iPSCs, compared to hESCs, is the hope to generate patient specific cells, where somatic cells such as skin cells from a patient could be utilized. The main benefit to this would be that men and women lacking egg and sperm, for any reason, could potentially get the opportunity to conceive children that are biologically their own. The major obstacle for the moment is the technical difficulties deriving a sufficient amount of iPSCs from differentiated cells without using a virus and/or risking epimutations in the host genome.

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Chapter 38

# Artificial ovary

Christiani A. Amorim

## Introduction

The reader may feel it is somewhat fanciful to see a chapter on artificial ovary assembly for further transplantation, when no studies have been published on this topic to date, and no one has yet been able to endorse its feasibility. However, we believe it is the right time to consider the artificial ovary as a promising technology in reproductive medicine, as it could represent an alternative to ovarian tissue transplantation. In addition, positive results with different types of artificial organs and tissues, such as bone, cartilage, skin, heart, liver, kidney, brain, cornea and teeth, indicate that construction of an artificial ovary is wholly achievable. Since there are no available studies on artificial ovary assembly for grafting purposes, this chapter may provide the starting point for this technology, where indications, advantages, strategies, techniques and possibilities will be discussed.

## The artificial ovary as a strategy to re-establish fertility

### Indications for patients

For cancer patients of childbearing age, fertility restoration often becomes an important concern after disease remission. In women, treatments such as chemo/radiotherapy can be very harmful to the ovaries, often causing loss of endocrine and reproductive functions, which results in premature menopause and emotional distress. An option for these patients is utilization of primordial follicles enclosed in ovarian cortex, since they represent 90% of the ovarian follicle reserve and there are no oncological, legal or ethical restrictions to their use. Before a patient begins cancer treatment, a sample of her ovarian tissue containing primordial follicles can be removed and cryopreserved. Then, after disease remission, the tissue fragments can be grafted back to the patient. This technique has led to successful ovarian function restoration, as well as pregnancy (for a review, see Donnez *et al.* [1]).

Transplantation has so far been the only option to re-establish ovarian function from cryopreserved ovarian tissue in cancer survivors. Despite the promising results mentioned above, there is a legitimate concern about the possible presence of malignant cells in the frozen–thawed fragments, which could provoke a recurrence of the primary disease after reimplantation [2]. Although many types of cancer never metastasize to the ovaries, leukemia is systemic in nature and poses a greater threat to the patient, while breast cancer is classed as moderate risk. Therefore, for patients diagnosed with these types of cancer, transplantation of ovarian tissue after disease remission is not advisable. Unfortunately, for these women, it is not yet possible to transplant their tissue after their health is restored.

A safe alternative for leukemia and breast cancer patients would be utilization of isolated follicles. Since the basement membrane encapsulating the ovarian follicle excludes capillaries, white blood cells and nerve processes from the granulosa compartment [3], grafting fully isolated follicles could be considered safer for these patients. Another option to avoid cancer re-introduction could be purging isolated follicle suspensions of malignant cells with specific antibodies [4]. Since it is likely that stromal cells are required for follicular growth, fully isolated follicles could be grafted together with autologous stromal cells (from a new ovarian biopsy after cancer treatment) [5].

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## Advantages

Although the main concern is being able to provide an alternative to restore fertility in patients who cannot benefit from ovarian tissue transplantation, the assembly and grafting of an artificial ovary would offer additional advantages compared to transplantation of ovarian tissue.

### Improvement of follicular survival by decreasing the ischemic period

After removal, freezing, thawing and transplantation, ovarian tissue is subjected to hypoxia in the first days post-grafting, and this deprivation of oxygen and nutrients, as well as accumulation of metabolic waste, may lead to cellular damage. Indeed, it has been estimated that a significant percentage (50–95%) of primordial follicles may be lost due to ischemia [6–8], which would directly affect the life span of the graft. Therefore, the success of primordial follicle transplantation depends on the growth of new blood vessels in order to restore adequate perfusion. This may be improved by use of a scaffold with a porous structure, loaded with factors known to promote angiogenesis. An interconnected pore network in a scaffold has been shown to enhance vascularization in prostheses implanted in the abdominal aorta of rats [9], while appropriate pore size and distribution would facilitate the diffusion of metabolites, oxygen and growth factors [10], which would have a positive effect on follicle survival and development. Angiogenic factors could also be added to the scaffold, either chemically immobilized or physically entrapped [10]. Shi *et al.* conducted studies to develop an artificial dermis and showed that when angiogenin, a polypeptide involved in angiogenesis, was added to a porous collagen-chitosan scaffold subcutaneously grafted to rabbits, vascularization increased [11]. Basic fibroblast growth factor (b-FGF) was also shown to have a positive effect on vascularization in different studies. Peters *et al.* observed almost fourfold faster vascularization when polylactic-co-glycolic acid (PLGA) microspheres were loaded with b-FBF. These authors reported that released b-FGF induced the formation of large and mature blood vessels in scaffolds implanted in the mesenteric membrane of rats [12]. Tanihara *et al.* also described induction of angiogenesis by b-FGF in heparin/alginate scaffolds grafted to the dorsal area of rats [13]. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) were found to increase vessel density, size and maturity when added to scaffolds grafted to mice [14]. According to Bergmann and West, these factors could well influence the differentiation of mesenchymal stem cells from the bloodstream into endothelial cells and encourage microvascularization [10].

### Control of follicular development

Premature recruitment of primordial follicles has also been suggested after grafting of ovarian tissue, possibly caused by a deficiency in inhibitory mechanisms implicated in the quiescence of primordial follicles in intact ovaries [15]. This is indeed very likely, since tissue collected for cryopreservation and transplantation comes from the ovarian cortex, where the vast majority of the follicular population is made up of primordial and primary follicles and there is a lack of larger follicles responsible for the production of inhibitory factors like anti-Müllerian hormone (AMH) and activin A. Using a scaffold, follicular activation and development may be modulated through supplementation of inhibitory and growth factors implicated in the different stages of folliculogenesis. These factors could, for example, be added encapsulated in materials with different degradation rates, according to requirements after transplantation.

### Improvement of follicular growth using fresh ovarian cells

Apart from the interaction between granulosa cells (GCs) and oocytes, follicles require neighboring stromal cells to support their growth. These cells are recruited to differentiate into theca cells, which play an essential role in follicular development through secretion of androgens as well as improving perifollicular vascularity. Although freezing of ovarian tissue does not negatively affect the morphology or ultrastructural characteristics of primordial follicles [16], it is harmful to surrounding tissue, causing damage to the extracellular matrix (ECM) and stromal cell necrosis [17], resulting in large areas of fibrosis [18]. The poor cellularity of tissue after freezing may influence the development of follicles, and could be involved in the lack of a structured thecal layer around secondary follicles and asynchrony between oocyte and follicular cell growth [19]. Therefore, in order to improve follicular development in the scaffold, a fragment of ovarian tissue could be removed before the scaffold is grafted, with the aim of isolating fresh stromal cells. These cells

would then be combined with isolated follicles from frozen tissue and seeded in the scaffold.

#### Control of the number and quality of follicles to be grafted

Due to the random distribution of primordial follicles in ovarian cortex, it is not possible to determine the number, or even the presence, of follicles in ovarian tissue to be transplanted to a patient [20], which could affect the life span of the graft. Grafting isolated follicles would allow not only the introduction of a high and known number of follicles into the host [21], but also assessment of follicular quality before grafting [22].

## How to assemble an artificial ovary

As previously mentioned, there are no studies on the construction of an artificial ovary for further transplantation and it is therefore necessary to start from scratch, studying the ovary and its characteristics to identify the main requirements, and analyzing studies on the assembly of other artificial organs, applying tissue engineering strategies to meet these requirements.

### The natural ovary

Although an understanding of mechanisms involved in folliculogenesis is essential to "build" an artificial ovary for transplantation, this process will not be discussed in the present chapter, since it can be found in several well presented review papers [23–25]. Nevertheless, it is important to bear in mind some basic information about the ovary.

The ovary has two main functions: production of oocytes capable of being fertilized, and secretion of hormones required by the reproductive tract during oocyte fertilization and further pregnancy [25]. Both functions depend on folliculogenesis, a complex developmental process that is regulated by various endocrine, paracrine and autocrine factors [26, 27], as well as intraovarian cell–cell and cell–matrix connections [3, 28]. Depending on developmental stage, follicles can be found in the outer or inner area of the ovary. While the cortex contains the vast majority of ovarian follicles, mostly in their initial stages of development, the medulla is highly vascularized and responsible for the maintenance of larger follicles.

In addition to follicles, different types of cells can be found in ovarian tissue, such as epithelial, stromal, endothelial and theca cells. As previously stated, these cells play an important role in the survival and development of follicles through the production or exchange of numerous factors essential for follicular quiescence, nutrition, communication, growth, hormone production, oocyte maturation, etc. Furthermore, ECM proteins synthesized by some of these cells serve as a scaffold to hold the ovary together and maintain the three-dimensional (3D) morphology of follicles. This 3D structure is indispensable to preserve intercellular interactions between granulosa cells and oocytes, regulating of many aspects of oocyte growth and development [29, 30].

### Requirements to assemble an artificial ovary

As in case of a natural ovary, the main goal of an artificial ovary is to offer an environment that allows folliculogenesis to occur. Therefore, just like the natural organ, the scaffold should: (1) ensure proper communication between follicles and ovarian cells; (2) preserve their interaction with the ECM; (3) supply factors involved in follicular survival and development; and (4) maintain the original structure of follicles. In other words, the scaffold should spatially and temporally mimic the ECM, the natural scaffold of the ovary. In order to do so, it should include some design parameters, such as interaction with cells, physical support of follicles, porosity, bioactivity, vascularization and biodegradability, which are all interconnected and influence each other. It must also be biocompatible and, from a practical point of view, capable of being handled.

#### Interaction with cells/follicles

Citing von der Mark *et al*., "Cells are surrounded by a wealth of information provided by the ECM, which presents adhesive and bioactive peptide epitopes located in matrix macromolecules and smaller glycoproteins, plus growth factors and cytokines trapped and sequestered by the matrix" [31]. The ECM thus plays an essential role in cell fate: it regulates cell morphology, proliferation, migration, differentiation, orientation, production and secretion of molecules and even death. For this reason, the scaffold should modulate the interactions of cells and follicles, supporting cell adhesion, proliferation, migration and production of matrix proteins necessary to form a substrate for new cells required for follicular development. For follicles, the scaffold should act as a supporting matrix, preserving their original 3D structure and intercellular

interactions between GCs and oocytes, which is essential to regulate follicular growth and development. The 3D arrangement of follicles is also influenced by cell migration and proliferation, induced by interaction of the scaffold and cells.

In order to modulate cell adhesion, behavior and function, polymer properties, such as crystallinity, morphology and surface, can be modified. Some of these important properties are discussed below.

#### Crystallinity

Crystallinity refers to the degree to which molecules of a polymer are oriented toward a repeating model. It is therefore favored by polymers with a chemically simple structure. Crystallinity may influence the response of cells to the scaffold, since it affects several surface characteristics, such as polarity and irregularity [32]. Degirmenbasi *et al.* reported that growth rates of fibroblasts varied according to the crystallinity range of poly(L-lactide) (PLLA); when the polymer was highly crystalline, fibroblasts showed lower growth rates [33].

#### Morphology

Scaffold topography and dimensionality play an important role in cell behavior. Topography can influence the morphology of cells, which consequently affects their orientation, proliferation, gene expression and function. Studies have shown that cells behave in a completely different manner when cultured on irregular surfaces (e.g. edges, grooves and ridges) rather than smooth surfaces [32, 34]. For example, ridges can influence cell orientation and migration of different types of cells. This cellular locomotory response is termed contact guidance. Regarding dimensionality, preservation of 3D structure is not only important for follicles, but also for cells. A 3D scaffold would provide normal polarity and spatial regulation of cells, and it may also influence the cellular response to existing physical and biochemical signals [32].

#### Surface

In tissue engineering, adhesion of cells to the scaffold surface is crucial because their activities (proliferation, migration, differentiation, etc.) depend on this. In order to improve cell attachment and thereby its survival and behavior in the material, modifications can be made to the scaffold surface. The advantage of treating polymer surfaces is that only the external part of the material is changed, preserving the polymer bulk. This is extremely useful, since materials with satisfactory bulk properties usually do not have the surface characteristics required for clinical application [35]. In this way, different polymers can be combined for specific purposes (i.e. surfaces with superior biocompatibility and bulk, with suitable mechanical properties).

Methods such as addition of adsorbed proteins and immobilized functional groups have been proposed to alter the surface chemistry of scaffolds. Polymer surfaces can be pre-treated with matrix proteins (fibronectin, collagen, laminin, etc) to increase cellular adhesion, which affects scaffold biocompatibility. Although ECM proteins show high adhesiveness and adsorb to practically all polymer surfaces, they may induce an immunoresponse in the host, since they are usually prepared from animal tissues. An alternative could be isolation and purification of these proteins, but the procedure is time-consuming and very expensive [31].

Incorporation of small biologically active functional groups, for example oligopeptides, saccharides and glycolipids, has also been attempted with the aim of isolating some features of ECM molecules associated with cell adhesion [34]. Some amino acid sequences, such as Arg-Gly-Asp (RGD), which is the cell-binding domain of fibronectin, play an essential role in cell binding and mediate cell adhesion. RGD is not exclusively linked to fibronectin; it can also be found in several ECM proteins, such as collagen, vitronectin and laminin. In addition, Tyr-Ile-Gly-Ser-Arg (YIGSR) and Ile-Lys-Val-Ala-Val (KVAV) laminin sequences also show cell binding activity and appear to mediate adhesion in some cell types [34].

Alternatively, chemical groups can be added through plasma, which consists of highly excited atomic, molecular, ionic and radical species [35]. Plasma treatment has been used to attach chemical groups or atoms to material surfaces. Use of oxygen plasma, for example, has been shown to increase the hydrophilicity of materials [35], which in turn increases material biocompatibility. Addition of chemical groups usually changes surface wettability and, consequently, improves cell adhesion [34]. Wettability can be defined as the ability of a liquid to adhere to a solid and spread over the surface. The surface wettability of a material is a measure of its hydrophilicity, hydrophobicity and surface-free energy [32], and it is associated with the crystallinity and chemical composition of the material [36]. Studying different types of mammalian cells, in 1978,

Grinnell demonstrated that surface wettability affects cellular attachment and proliferation, and adhesion favors water-wettable substrates [37]. Biocompatibility is also a vital parameter associated with surface hydrophilicity. One can therefore assume that, in order to improve cell adhesion and scaffold biocompatibility, hydrophilic materials should be used. However, this type of material usually has poor mechanical integrity and is often utilized as a coating for a more robust and less hydrophilic polymer. For instance, poly(ethylene oxide) (PEO) has been used to create hydrophilic surfaces that become easily hydrated [32].

### Physical support for the follicles

As previously stated, for folliculogenesis to occur, it is necessary to maintain contact between GCs and oocytes because many aspects of oocyte growth and development are regulated by interactions with adjacent GCs [29, 30]. A rupture in the GC–oocyte connection would lead to uncoordinated growth and differentiation of somatic and germ cells [38]. In order to avoid breakdown of the metabolic link between GCs and oocytes, follicles need to maintain their 3D structure. Three-dimensional scaffolds would be able to effectively mimic physiological conditions, since many cellular processes in organogenesis occur exclusively in 3D [39]. Previous studies on in vitro culture of isolated human follicles have shown that preservation of their 3D structure positively affects their survival and growth [20, 40].

Although the scaffold needs to be able to support the 3D structure of follicles, it should not be excessively stiff to prevent their exponential growth. An extremely rigid scaffold may decrease the proliferation rate of GCs and oocyte growth, and affect actin organization in growing follicles [41], which could lead to diminished follicular growth and even apoptosis.

### Porosity

Scaffolds of appropriate size with good pore distribution and interconnectivity are essential for uniform cell seeding and distribution and tissue ingrowth. Porosity also influences invasion and migration of cells from the host, which positively affects biodegradation and neovascularization of the scaffold. In addition, an interconnected pore network is necessary for transportation of signaling molecules, growth factors, oxygen and nutrients, as well as metabolic waste removal.

Another parameter to bear in mind is follicular diameter. Human primordial follicles have a diameter of around 30 μm and it is important to ensure correct pore size in order to allow easy follicle penetration and distribution in the scaffold. However, one should be cautious in this regard because very large pores decrease the surface area available for cellular attachment, consequently slowing tissue ingrowth, and reduce the mechanical properties of the scaffold due to increased void spaces [10].

### Bioactivity

In order to assemble an artificial ovary, the scaffold should act as a vehicle to graft isolated follicles, ensuring preservation of their 3D structure. However, it should also have a bioactive function, regulating the growth of these follicles. It is known that many biologically functional molecules, ECM components and cells interact on the nanoscale, creating a highly specialized microenvironment that is essential for proper cell development and continued function. For this reason, in order to induce and coordinate folliculogenesis in a grafted scaffold, it is necessary to program it to deliver bioactive molecules, such as factors that may positively influence neovascularization, follicle growth and development and oocyte maturation. These factors could be added using polymeric systems, which would allow regulation of the localization, duration, delivery and availability of different inhibitory and growth factors [42]. To this end, several strategies have been applied, such as multiple levels of encapsulation, noncovalently bonding bioactive factors to peptides with a range of dissociation constants that mimic the immobilization of growth factors in the ECM [43]. Encapsulation, for example, can protect these factors from denaturation that could occur if they were directly adsorbed onto the scaffold, which would result in their complete degradation in a very short space of time. The released quantity of factors may be modulated by the encapsulated quantity in the microspheres, by the number of microspheres incorporated in the scaffold or by the composition of the microspheres. Thus, microspheres containing different factors implicated in folliculogenesis, factors mitigating ischemic damage, as well as factors involved in angiogenesis can be tested.

### Vascularization

Vascularization is a crucial element in the success of a scaffold, required to supply oxygenation and nutrients to grafted cells and follicles and remove metabolic waste. However, formation of new capillaries in the

material is very challenging, as it necessitates interaction of different design properties, such as choice of material, porosity and pore interconnectivity, bioactivity and biodegradation. When follicles and/or cells are grafted with a scaffold, they are only oxygenated by simple diffusion, which may be limited by the construct [10]. This oxygen and nutrient deprivation may cause cellular damage and apoptosis. An additional concern is that neovascularization may be slower than cell proliferation and follicle development in the grafted scaffold, creating a higher demand for oxygen and nutrients than the new capillaries can meet, which can also lead to follicular death.

Several strategies can be applied to promote vascularization. As for transplantation of ovarian tissue fragments, the scaffold can be grafted to a peritoneal window close to the ovarian vessels and fimbria. This window should be created a few days earlier in order to induce angiogenesis and neovascularization in the area destined for scaffold grafting. Alternatively, the cortex of the ovary remaining after cancer treatment could be removed to place the scaffold in direct contact with the medulla, an extensively vascularized area (for a review, see Donnez *et al.* [1]). Grafting of autologous endothelial cells may also be an option to promote vascularization in the scaffold, as well as addition of growth factors involved in neovascularization (bFGF, VEGF, PDGF), as previously discussed (for a review, see Bergmann and West [10]).

### Biodegradability

Ovarian follicles are exceptional in that they can grow to around 600 times their size during folliculogenesis (the human follicle grows from 30 μm in its primordial stage to 18–24 mm when it is ready to ovulate). In addition, they recruit cells and vessels to support their development. An ideal scaffold would need to degrade in order to allow exponential growth of follicles, formation of vessels, and proliferation of stromal cells. Ideally, the artificial ovary should offer an appropriate initial environment for follicles that would be replaced by a new "ovarian-like" structure after a few weeks of grafting. The degradation rate of the scaffold is thus an essential parameter in the success of grafting.

Although biodegradation should be conceived with follicular development in mind, other factors should also be taken into account. The biodegradation rate cannot be faster than cell migration and proliferation or ECM synthesis and stabilization in the scaffold, because cells would lose physiochemical factors for tissue regeneration [10] and isolated follicles would lose their 3D support. On the other hand, slower degradation would inhibit cell penetration and consequently ECM formation in the scaffold [10] and negatively affect follicular growth. Therefore, the material should be carefully selected in order to control the desired degradation rate.

Degradation of the material is characterized by different parameters – loss of molecular weight, loss of mass or loss of mechanical strength [44] – and is associated with its molecular weight, wettability and crystallinity [10]. Knowledge of these parameters is therefore rooted in the comprehension of the degradation rate of different polymers, which is fundamental to choosing a material that will match the desired degradation kinetics.

The scaffold should also degrade into products that can be easily eliminated through metabolic pathways [36]. This is very important when considering the biocompatibility of the material [36], since these products cannot be toxic to cells around the scaffold or organs of the lymphatic system [10]. For instance, polylactic acid (PLA) and polyglycolic acid (PGA), which are among the few degradable polymers approved for human clinical use by the US Food and Drug Administration (FDA) and have a clinical application as sutures, are known to decrease pH in the area surrounding the scaffold during their degradation. Such acidity has been implicated in adverse tissue reactions [44] due to the inflammatory response of surrounding tissue.

### Biocompatibility

Biocompatibility is one of the most important characteristics of a scaffold and it has been shown to be related to some of the above-mentioned parameters, such as the degradation rate and byproduct and material wettability. Material biocompatibility can be evaluated by measuring the duration of adverse variations in the homeostatic mechanisms that determine the host response [45].

Scaffold grafting inevitably causes damage to surrounding tissue, inducing an inflammatory response, foreign body reaction and cascade of wound healing [45]. Depending on the magnitude of the inflammatory response triggered by the scaffold, the migration of cells such as macrophages and neutrophils to the grafting site may have undesirable effects on the success of the scaffold. These cells, involved in the inflammatory reaction, can secrete enzymes that increase the degradation rate of the scaffold, and molecules that

block activation of factors essential to the survival of cells in the scaffold [10]. In addition, a strong inflammatory response may also trigger the repair mechanism, which would result in the formation of scar tissue and a fibrous capsule around the scaffold. This would negatively affect its function and lead to death of cells and follicles present within.

Biocompatibility is usually excellent with natural polymers, such as polysaccharides and proteins, since their structure is very similar to the native cellular environment, and with hydrogels because of their high water content, which also mimics the natural ECM.

### Scaffold handling

Having a scaffold lacking the mechanical strength to be handled during surgery would not be ideal. For instance, although hydrogels have the advantage of mimicking the natural ECM, they usually do not have adequate mechanical strength and may be damaged or even destroyed during handling.

Mechanical strength is affected by scaffold composition, pore size and porosity. Therefore, while hydrophilic and very porous materials are probably the best choice for grafting isolated follicles, the effect of these parameters on the mechanical properties of the scaffold should also be taken into consideration.

## The tissue engineering approach

In recent decades, scientists from all over the world have been developing strategies to assemble different types of artificial organs and tissues. At first, the idea was to physically replace the lost structure, but increasingly, the metabolic function of tissues has been taken into account. This has involved various tissue engineering strategies and, in the last decade, the construction of artificial organs has emerged as a leading domain in this revolutionary and exciting new field.

Tissue engineering is a multidisciplinary field, incorporating different areas of study, such as biochemistry, surgery, engineering, physics and physiology, thereby combining principles of life science with material science. It aims to create new, or restore damaged or malfunctioning, tissues or organs [46] through the introduction of biological products (e.g. proteins and cells) in to a synthetic or natural matrix able to support and organize them [34]. In this context, tissue engineering has been responsible for the creation of many different materials capable of mimicking tissues and organs.

Among the many applications of tissue engineering, the most important for artificial ovary technology is the possibility of having a ready-made substitute for the ovary by seeding the patient's own cells and follicles to a biodegradable scaffold, with a view to tissue regeneration or construction of a new ovary. The question of scaffold manufacture is therefore crucial. Fabrication approaches must not only replicate the properties of the ovary at the macroscopic level, but also recreate nanoscale details observed in native tissue at the cellular level. Different synthetic and natural polymers can be used for this purpose. Although natural polymers, like hyaluronic acid, poly γ-glutamic acid and collagen, exhibit high biocompatibility and biodegradability, they may pose a risk of antigenicity and show variations according to batch production. On the other hand, synthetic polymers, such as alyphatic polyesters, polyanhydrides, polypropylene fumarates and polyphosphazenes, offer outstanding reproducibility, the ability to control degradation kinetics, and high mechanical strength [47]. However, they may show low biocompatibility compared to natural substances.

To process the different materials into porous scaffolds, several techniques have been developed [46, 48–50]:

- **Gas foaming** – A biodegradable polymer is saturated with carbon dioxide ($CO_2$) at high pressures. The solubility of the gas in the polymer is then decreased rapidly by bringing the $CO_2$ pressure back to atmospheric levels. This results in nucleation and growth of gas bubbles.
- **Fiber bonding/fiber meshes** – This technique increases the mechanical properties of a scaffold by dissolving PLA and casting it over a PGA mesh, for example. The solvent is allowed to evaporate and the construct is then heated to exceed the melting point of PGA. Once the PLA–PGA construct has cooled, the PLA is removed by dissolving it again. This treatment results in a mesh of PGA fibers joined at the crosspoint.
- **Phase separation** – The polymer solution separates into two phases, a polymer-rich phase and a polymer-lean phase. After the solvent is removed, the polymer-rich phase solidifies. Biologically active molecules can be added to the polymer solution.
- **Melt molding** – One of the techniques involved in this process involves filling a Teflon mold with

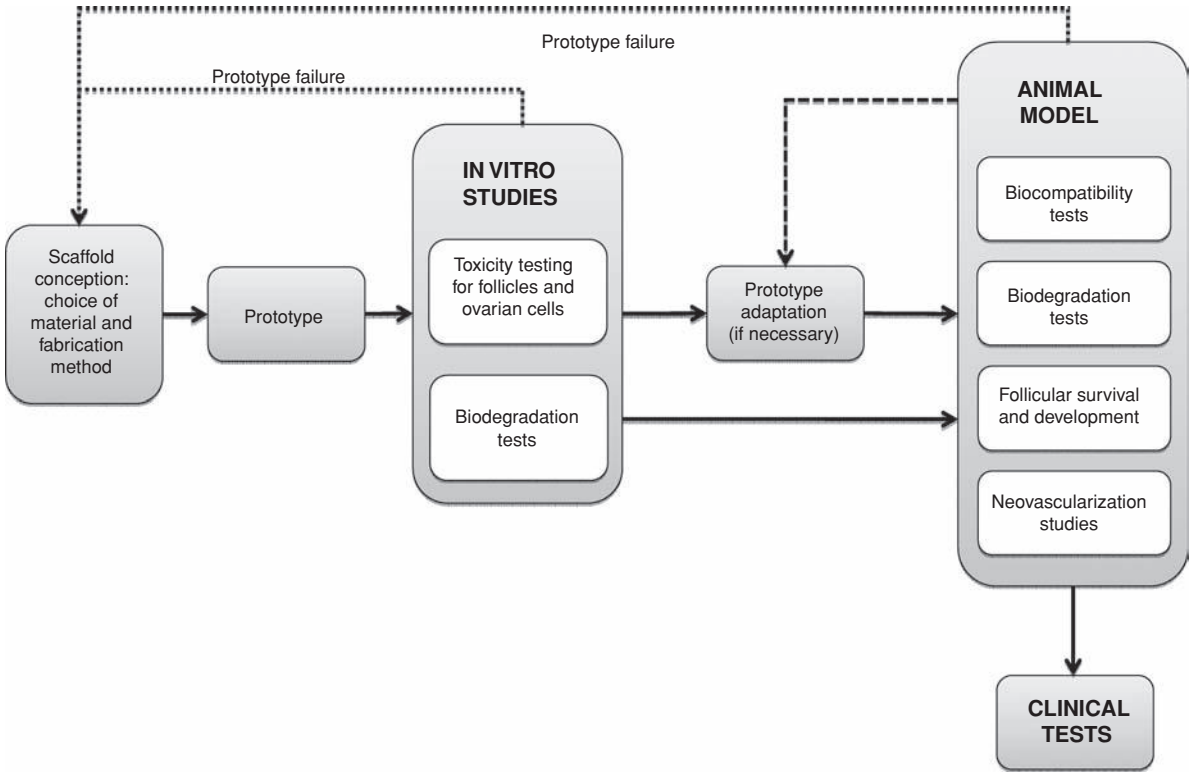


**Figure 38.1** Schematic approach to the development of an artificial ovary.

polymer powder and gelatine microspheres of specific diameter, and then heating the mold to above the glass-transition temperature of the polymer, while applying pressure to the mixture. This treatment causes the polymer particles to bond together. Once the mold is removed, the gelatine component is leached out by immersing the scaffold in water, before drying it.

- **Emulsion freeze-drying** – This process involves adding ultrapure water to a solution of methylene chloride with PGA. The two immiscible layers are then homogenized to form a water-in-oil emulsion, which is quenched in liquid nitrogen and freeze-dried to produce the porous structure.
- **Freeze drying** – The polymer is dissolved in glacial acetic acid or benzene and the resultant solution is frozen and freeze-dried to yield porous matrices.
- **Solution casting** – The polymer is dissolved in chloroform and then precipitated by the addition of methanol before the material is pressed into a mold and heated to 45–48°C for 24 h.
- **Solid freeform fabrication techniques** (also known as rapid prototype) – These are computer-controlled fabrication techniques that create complex scaffold designs, with localized pore morphologies and porosities, as well as incorporated bioactive molecules to suit cell requirements. The general process involves producing a computer-generated model using computer-aided design (CAD) software. This CAD model is then expressed as a series of cross-sectional layers. The data are then fed to the solid freeform fabrication machine, which produces the physical model.
- **Indirect solid freeform fabrication technique** – In this procedure, a negative mold is generated by one of the solid freeform fabrication techniques and the scaffold is formed by adding the casting solution to the negative mold using the desired polymer. After solidification, the negative mold is removed by dissolution.
- **Particulate-leaching** – With this technique, salt is first ground into small particles and those of the

desired size are transferred into a mold. A polymer solution is then cast into the salt-filled mold. After evaporation of the solvent, the salt crystals are leached away using water to form the pores of the scaffold.

- **Electrospinning** – This is a process capable of producing ultra-fine fibers by electrically charging a suspended droplet of polymer melt or solution.
- **Vibrating particle fabrication technique** – In this process, the polymer is dissolved in solvent and the solution is molded with salt particles. The particles are dispersed using vortex and, at predetermined time intervals, more particles are added. The solution then evaporates under continuous vibration and the scaffold is subjected to heat and vacuum.

## Conclusion

Having provided a comprehensive description of the assembly of an artificial ovary and the involvement of tissue engineering strategies, we find ourselves at the starting point of a new technology that may be termed "ovarian tissue engineering." The aim of this strategy is to replace not only the structure of lost tissue, but rather its function, to potentially allow endocrine activity and fertility to be restored in cancer patients.

Ovarian tissue engineering research should explore different alternatives, involving a variety of materials that can be tested alone or in combination, techniques that can turn these materials into functioning scaffolds, and many other factors that may be combined to give these scaffolds optimal bioactive properties. This will require numerous in vitro and in vivo experiments on animal models, with data quantifiable by functional and structural endpoints (Figure 38.1). It is therefore likely that this innovative approach will prove very challenging, probably taking many years or even decades to achieve successful results. However, since it may represent a viable option for re-establishing fertility in cancer patients, it should be extensively investigated.

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Chapter 39

# Predicting ovarian futures

## The contribution of genetics

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## Introduction

One of the consequences of cancer therapies, including radiation and chemotherapy, is gonadotoxicity. As effective treatments have rendered a number of malignancies curable, or have delivered long-term survival, post-treatment fertility has emerged as an important consideration for patients and their healthcare providers. Unfortunately, there are currently no definitive ways to limit the injurious effects of these treatments on gonadal function, other than shielding the gonads from direct exposure to ionizing radiation. Suppression of gonadotropin secretion may have a protective effect in some populations, but the efficacy of this intervention for preserving fertility remains to be conclusively established.

The banking of germ cells or embryos prior to treatment represent options for preservation of fertility. Sperm cryopreservation is a longstanding option for sexually mature males, and embryo cryopreservation is an option for some women and couples. Though recent successes with cryopreservation of ovarian cortex or oocytes are encouraging, options for gamete or embryo preservation are more complicated for women, and entail procedural risks and expense. Moreover the technologies have not advanced to the point that female options for fertility preservation are as successful in outcomes as pre-treatment sperm cryopreservation. The decision to pursue ovarian cortex or oocyte banking is complicated by the fact that the gonadal response to radiation and chemotherapy varies among the population, and there is, at present, no precise way to determine who will suffer irreversible damage and who will emerge from treatment with fertility intact, obviating the need for pre-treatment interventions. Additionally, preserving the ovarian germ cell complement per se, while an important determinant of fertility, does not necessarily insure it. Furthermore, the inability to make predictions regarding the extent of post-treatment gonadal function impacts the design of research on interventions to spare fertility. Evaluations of such interventions could be conducted more efficiently with smaller sample sizes based on a more precise knowledge of subject risk for significant post-treatment gonadal dysfunction. In this chapter, we provide a framework for thinking about factors that can predict gonadal function post-cancer therapy, focusing on genetic contributions and reviewing outstanding issues that need to be addressed in future research with the aim of developing patient-specific algorithms that are predictive of post-treatment fertility or infertility.

Predicting the weather requires knowing what the current weather conditions are in the specified location such as air temperature and humidity, what fronts and air masses are approaching and what other conditions might affect them. Local geography and previous weather patterns must also be taken into consideration. These are all elements that a meteorologist must assess before predicting future weather conditions. Using the weather prediction rubric, we can identify four general requirements for accurately predicting a future biological event such as preservation of ovarian function after cancer therapy:

1. The stability or flux of the current condition must be known (i.e. baseline fertility, germ cell complement and rate of germ cell depletion).
2. Knowledge of what events or conditions could change the stability or rate of flux (i.e. types of treatment, dosing, duration).
3. The likelihood of those events or conditions happening (i.e. epidemiological data on

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post-therapy fertility by types of agents, dose and treatment duration and age-related effects).
4. Potential for interactions of those events affecting outcome (i.e. host variables including differences in drug metabolism and action, intrinsic factors [i.e. genes] affecting follicular complement, co-morbidities).

## The current state of prediction of future ovarian function following cancer therapy

Alterations of the rate of follicle loss have been investigated in patients undergoing radiation and chemotherapy. However, other than age of the patient and dosing of agents, specific predictors related to infertility have not been established. The current American Society of Clinical Oncology's categorization of risk for gonadal dysfunction is broad and descriptive: (1) low; (2) medium; and (3) high. The medium-risk category encompasses risk of permanent cessation of menses (used as a surrogate marker of ovarian function) of between 30 and 80% [1]. The large range of probability in the medium-risk category is frustrating to physicians counseling patients; it also strongly suggests that there is substantial individual variation to ovarian susceptibility to damage by radiation and chemotherapy. Thus, while useful in understanding the effects of toxic agents on ovarian function, the existing classification of risk is not at all useful in predicting an *individual's* future ovarian function.

## Genetic risk factors for early ovarian senescence that could impact ovarian function after cancer treatment

In the last decade there has been a body of accumulated work that has identified genes that have a role in placing women at risk of earlier ovarian senescence. It is plausible that these risk alleles place women at an even greater risk of infertility after cancer treatment, although this notion has not been experimentally evaluated. Nonetheless, the wide range of ovarian compromise seen in women who have been treated for cancer, even with the same therapies, strongly suggests that the individual genetic variation controlling ovarian function may play a role in determining the level of ovarian damage.

Eggs reside in groups of cells called follicles within the ovary. Except for late in the reproductive life span, the majority of follicles are in a resting state at the primordial stage of development. The complement of primordial follicles has been called the resting pool [2] and represents ovarian reserve or the population of oocytes that have not yet become committed to the path of growth and ovulation. Since follicles (and eggs) are progressively lost from the resting pool over time by entering the growing pool or undergoing apoptosis, the ovarian reserve is constantly declining over time in women. In general, women with a diminished ovarian reserve are considered to have reduced fecundity [3].

Decay curves modeling the decline of the resting pool of follicles have been presented in the literature in mice and humans [4, 5]. In women a bimodal decay was described, with a sharp increase in the rate of follicle loss at an average age of 38 years. However, a more recent model suggests that the rate of follicle loss or resting pool decay is one of slight constant acceleration throughout the perimenopause [6]. Recently, investigators have explored the impact that radiation and chemotherapy have on shifting this "follicle population decay curve" to the left [7]. This model describes diminished ovarian reserve ensuing at an earlier age after the toxic treatments.

The ability to predict the consequences of decay in the germ cell population depends upon determining a woman's current ovarian reserve, and there is presently no reliable method to accomplish this task. Antral follicle counts can vary in the hands of different observers, and biomarkers such as anti-Müllerian hormone and inhibin B suffer from the lack of standardized assays and insufficient normative data. Moreover, predicting how that reserve might change over time is even more challenging.

There are several environmental and genetic conditions with clearly defined deleterious effects on ovarian reserve, including exposure to environmental/occupational toxins like 4-vinylcyclohexene diepoxide, heavy tobacco use, specific X-chromosome deletions and genetic variants (e.g. *FMR1* permutations). When present, these might reasonably be expected to increase risk of ovarian failure following exposure to a gonadotoxic therapy. However, there are large number of genes involved in human ovarian development and follicular growth and their potential contributions to variability in response to cancer therapies are largely unknown. There are also other factors that could affect ovarian function when compounded with a gonadotoxic cancer therapy, whose impact is less well understood including exposure to

bisphenols, dioxins, aniline dyes, immune issues or even body mass.

## Control of ovarian development may impact future fertility (Table 39.1 [3, 8–13])

Female fertility is dependant upon a series of critical ovarian developmental events that are controlled by a large number of genes on autosomes and the X chromosome. Variation in these critical genes which impact negatively on female fertility or fecundity might have even greater impact on women post-cancer treatment.

During ovarian development, primordial germ cells must establish themselves in the yolk sac endoderm and then migrate to the gonadal ridge. Once there, the presumptive oocytes must survive and become inhabitants of the cords of cells that will become granulosa cells. After the primordial follicle pool is established, it is at risk of diminution from both external and internal factors. Follicles can undergo destruction by toxins such as radiation or chemotherapy. Initiation of growth of larger groups (cohorts) of follicles can occur which would deplete the primordial pool more rapidly. However, to achieve fertility, follicles must grow in an orderly fashion in the proper endocrinologic milieu to allow ovulation of a mature fertilizable oocyte that can be fertilized and implant and grow within the uterus. Normal progression of folliculogenesis is just as important to continued ovarian function as maintaining ovarian reserve [8]. Anything that disrupts the process of gametogenesis from the initiation of embryonic germ cell formation to implantation of a fertilized embryo can reduce fertility.

Gallardo *et al.* investigated the mouse genome for candidate genes involved in murine ovarian fertility and identified 348 candidate genes involved in different stages of folliculogenesis [9]. No similar study has been performed in humans, but a number of genes have been clearly defined as being essential for human ovarian function and normal reproductive life span. However, a review of candidate genes associated with premature ovarian failure as well as linkage studies was recently presented by van Doren *et al.* [14].

## The X chromosome role in ovarian function

Human ovarian development requires the activity of autosomal genes and two functional X chromosomes.

**Table 39.1** Selected genes and proteins with a reported role in follicle formation and growth in mammals

| |
|---|
| **Germ cell differentiation** |
| • BMP-4<br>• Smad1<br>• Fragilis<br>• Stella<br>• GATA4<br>• PUM2<br>• DAZLA |
| **Germ cell migration and survival** |
| • c-kit<br>• kit ligand (SCF)<br>• SOX3<br>• BMP/GDF-9<br>• TGFβ<br>• TNFα<br>• LIF<br>• SDF-1<br>• CXCR4<br>• Laminin<br>• Fibronectin |
| **Germ cell proliferation** |
| • POG<br>• TGFβ<br>• TIAR<br>• FMR1<br>• PIN1 |
| **Gonadal formation and colonization** |
| • TIAR<br>• LIM (Lhx9)<br>• SF1<br>• WT1<br>• DAX<br>• FIGα<br>• FOXL2<br>• Cadherin<br>• $\beta_1$—integrin |
| **Oocyte-specific early maturation** |
| • Nobox<br>• IGF2bp2<br>• Maelstrom<br>• PTEN<br>• TSC1<br>• Foxo3a<br>• P27<br>• ZP 1–3 |
| **Follicle growth** |
| • Slc18a2<br>• Smad3<br>• FSHR<br>• HSD17b1<br>• Cyp11a1<br>• Hsd3b1<br>• Ihna<br>• Dax1<br>• Greb1<br>• LHx8<br>• Nrf2<br>• Gdf-9<br>• Mater<br>• AMH/MIS<br>• Activin<br>• BMP-15<br>• Fog2<br>• ESR2 |

References: [3, 8–13].

Although the requirement for two X chromosomes for ovarian development has long been recognized, the essential human X chromosome genes and their functions are largely unknown [10]. Cytogenetic studies have yielded clues as to the location of key ovarian function genes. Terminal deletions from Xp11 to Xp22.1 are associated with primary amenorrhea, and deletions from Xq13 to Xq27 are usually associated with primary amenorrhea or premature senescence [11]. The region encompassing Xq13 to Xq26 is considered to be the "critical region" and this domain has been subdivided into two subregions Xq13–21 ans Xq23–27 [15].

The study of X chromosome gene variants and mutations has yielded a number of candidates for loci controlling germ cell complement. Among these is the fragile X syndrome gene (*FMR1*) on Xq27.3, which encodes an RNA-binding protein. Premutations in *FMR1*, represented by increased numbers of CGG trinucleotide repeats in the 5'-untranslated region, are well established to be associated with premature ovarian dysfunction.

The human orthologue of *Drosophila* diaphanous 2 (*DIAPH2*), a gene located on Xq22, encodes a protein involved in cytokinesis that, when mutated, causes sterility in flies. Premature ovarian failure associated with a translocation that disrupted the *DIAPH2* gene has been reported. *XPNPEP2*, a gene located at Xp25, which encodes aminopeptidase P, was disrupted by a translocation associated with secondary amenorrhea. The zinc finger gene, ZFX, located on Xp22.1, is known to be important in murine ovarian development because heterozygous and homozygous mutations are associated with a reduced germ cell number. A mutation in the progesterone receptor membrane component-1 (*PGRMC1*) gene, located on Xq22-q24, has been found associated with premature ovarian failure. *BMP-15* located at Xp11.2 is a candidate gene for control of germ cell complement based on known ovine variants that result in follicular growth arrest in the Inverdale and Hanna sheep. However, genetic evidence for a role for *BMP-15* in human ovarian dysfunction is weak [16, 17].

## Autosomal genes and regions of interest in ovarian function research

Autosomal genes are also play important roles in controlling follicular dynamics. Mutations in the follicle stimulating hormone (FSH) receptor gene (*FSHR*), the ataxia telangiectasia gene (*ATM*), which is implicated in DNA repair and cell cycle control, the homeobox gene, *NOBOX*, *NR5A1*, also known as steroidogenic factor-1, a transcription factor in nuclear receptor family, and the forkhead transcription factor, *FOXL2*, are all associated with ovarian dysfunction or premature ovarian failure in certain populations. Mutations in *FOXL2* cause blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), the type 1 form of which is associated with premature ovarian failure [12].

Mutations resulting in intranuclear aggregation and cytoplasmic mislocalization of FOXL2 are predictors of ovarian dysfunction. Polymorphisms in the gene encoding Inhibin alpha subunit (INHA) have been associated with premature ovarian dysfunction in some studies, but others dispute this association [18, 19].

Mutations in the catalytic subunit of the mitochondrial DNA polymerase gene, *POLG*, have been reported to segregate with premature ovarian dysfunction and ophthalmoplegia [20].

There has been one genome-wide linkage scan in a Dutch family that identified a region on chromosome 5 as a possible locus for familial premature ovarian dysfunction [21]. Comparative genomic hybridization profiling in a group of 99 Caucasian women with premature ovarian failure [22] resulted in identification of 8 regions with copy number variations (1p21.1, 5p14.3, 5q13.2, 6p25.3, 14q32.33, 16p11.2, 17q12 and Xq28). It is evident that larger genomic studies conducted in multiple populations are needed to identify gene variants associated with premature ovarian dysfunction and mutations across biogeographical ancestry.

Genes that have been associated with early ovarian senescence observed in isolated family groups or individuals are listed in Table 39.2 [3, 10–13, 15–20, 23–29]. Although it may be assumed that women inheriting such genes or even groups of polymorphisms might have an earlier cessation of ovarian function, no markers have yet been shown to be predictive for *individual fertility*. There have been several promising recent studies correlating specific gene variants/mutations with *markers of diminished ovarian reserve* [14, 23, 30]. However, prospective studies validating these correlations are lacking at this time.

## Outstanding questions

In order to build a clinically useful patient specific prediction algorithm for post-treatment infertility, we

**Table 39.2** Genes associated with early ovarian senescence in humans

| Autosomal | X-linked |
|---|---|
| • **FOXL2** | • **FMR1** |
| • **NOBOX** | • GDF-9 |
| • **FSHR** | • FMR2 |
| • **AIRE** | • DIAPH2 |
| • **ATM** | • XPNPEP2 |
| • **POLG** | • BMP-15 |
| • **NR5A1** | |
| • MSH5 | |
| • NOG | |
| • INHA | |
| • FOXEI | |
| • B-glycan | |
| • PTHB1 | |
| • AR | |
| • DMC1 | |
| • ESR1 | |
| • PCMT1 | |
| • MCKDHB | |
| • ASCL6 | |
| • PGRMC1 | |
| • FIGLA | |
| • DMCI | |
| • SALL4 | |
| • PTEN | |
| • TGFMR3 | |

References [3, 10–13, 15–20, 23–29].
**Bolded** genes have strong evidence for a role in ovarian senescence.

need as a foundation the answers to several questions regarding individual determinants of fertility.

## Is fertility a heritable condition that can be predicted?

The inheritance of fertility has been studied in discrete populations by Blum *et al.* who used mitochondrial DNA samples from different human population groups to determined that the degree of imbalance of gene genealogy increases with fertility inheritance [31]. That such differences could be associated with fertility differences, suggests that genomic analysis might be useful in fertility prediction.

Although there are genes and markers that are associated with early ovarian senescence, there is limited data about the predictive value of testing for such information. But the discrete limit of ovarian function does create the desire to predict its course over time. This academic pursuit has had a renewed driving force from cancer survivors that want to predict accurately if they will be able to have children after their therapy. Should they delay cancer treatment to undergo expensive and unproven therapies to hold on to the possibility of future reproduction? Or should they get on with their therapy and know that in all likelihood they will continue to have ovarian function after chemotherapy or radiation?

The complexities of the events of fertility go beyond ovarian function. External factors such as individual choices in fertility management or choice of a partner also affect ultimate fecundity and fertility rates [32], as well as factors such as fertilization opportunities, uterine function and implantation events [33]. Since predicting fertility is fraught with confounding factors, it may be more practical clinically to attempt to predict the likelihood of infertility after agents destructive to oocytes are administered.

## Can age at normal menopause be predicted?

There are several studies underway as well as a few recent reports identifying genetic factors underlying normal menopausal age, considered to range from 40 to 60 years [24]. Polymorphisms of CYP1B1 and MTHFR have both been associated with timing of menopause [25, 26]. Linkage studies have identified loci on chromosome X as well as chromosome 9 with variations in age at natural menopause [24] (Table 39.3 [24, 34–36]).

Two recent genome-wide association studies have identified loci associated with influencing age at menopause. Stolk *et al.* identified 6 loci in European women associated with differences in age at menopause on chromosomes 12, 19 and 20 [34]. They predicted a 19% risk of menopause prior to age 50 with the loci at 19q13.4 and 20p12.3 and similar magnitude risk for increases in age at menopause for the other loci. He *et al.* analyzed patients of European ancestry from the Nurses Health Study and the Women's Genome Health study in the USA [35]. They found 4 regions of interest, 5q32.2, 6p24.2, 19q13.42 and

**Table 39.3** Loci associated with natural age at menopause

| | |
|---|---|
| 5q | 35.2 |
| 5q | 14.1–15 |
| 6p | 24.2 |
| 9q | 21.3 |
| 13q | 34 |
| **19q** | **13.42** |
| **20p** | **12.3** |

Compiled from references [24, 34–36].
**Bolded** loci were found in both studies [34] and [35].

20p12.3. The authors suggest that all 4 loci together account for 2.69% of the variation of age at menopause. Of note, two loci were identified by both studies (19q13.42 and 20p12.3).

Clearly, factors other than just the markers reported so far influence the age at menopause, including environmental exposures, medications and pregnancies. However genome-wide screening has been able to reveal novel loci and its further use may accelerate acquisition of knowledge of the genetic factors involved in the reproductive life span. Understanding which genetic factors are relevant will likely require a combination of hypothesis-driven evaluations based on surmised role of suspect genes in ovarian function and non-hypothesis driven whole genome surveys [3].

## Can menopausal timing determinants predict fertility?

Menopause on average occurs around age 52 years; however, natural fecundity ends upon average around age 41 years [33]. Thus, factors that control the cessation of menses may not directly correlate to an increase or decrease in fertility. It makes sense that factors that would result in earlier ovarian cessation would likely also result in earlier loss of fertility [28]. If such factors result in a constant but increased rate of loss of the resting pool of follicles, the left shift in the decay curve proposed by Wallace might be appropriate for predicting fertility as well as menopause. However, it cannot be assumed that all conditions that might affect age at menopause would necessarily also affect age at diminution of fertility. However the predictive ability of such proposed profiles will need to be clearly established before they can be used to advise patients clinically about risk profiles of their future fertility.

## What is the future of future fertility?

We have identified a number of questions and very few answers in predicting future ovarian function. But if we revisit the original four key elements of the weather prediction paradigm, a framework for predicting ovarian function begins to come into focus:

1. **The stability or flux of the current condition must be known.** Data is rapidly accumulating regarding serum and sonographic markers of current ovarian reserve. These markers have not yet been directly linked to predicting fertility, and validation and standardization of these assays are ongoing. Reliable assessment of an individual's current ovarian reserve will be invaluable in predicting future ovarian fertility.
2. **Knowledge of what events or conditions could change the stability or rate of flux.** Data is slowly accumulating, defining the diminution of ovarian reserve from various doses of chemotherapeutic agents though changes in chemotherapy combinations and dosing regimens make determinations of specific toxicities difficult. Radiation parameters may be more rapidly available [7].
3. **The likelihood of those events or conditions happening.** Individual sensitivity to chemotherapy toxicities is a topic under investigation in pharmacogenomics studies. Individual variations in chemotherapy metabolism are just as likely to vary in ovarian toxicity as lung, liver or bone toxicities. Specific risks for diminished ovarian function need to be better defined before their incidence can be assessed.
4. **Potential for interactions of those events affecting outcome.** There are no studies as yet that report the baseline ovarian function, behavioral or environmental risks, genetic markers and specific chemotherapy and/or radiation treatments correlated to fertility rates after treatment.

All of these will be necessary in establishing a useful paradigm for risk assessment can be applied to individual patients to predict ovarian function following cancer therapies.

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Chapter 40

# Psychological issues of cancer survivors

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## Introduction

Patients whose fertility would be impaired due to treatment for their medical condition face a daunting task and need timely, accurate information about their options. They need to know the short and long-term side effects of medical treatment on their reproductive system prior to initiating adjuvant chemotherapy. They may require help finding fertility specialists. Many of the currently available strategies to sustain fertility are far from certain to succeed and fertility-sparing options may carry their own risks. Cancer patients may need psychological support for emotional distress occurring because of the cancer diagnosis and/or for psychological distress which exacerbates prior emotional difficulties. This chapter will focus on what we know about the emotional needs of cancer patients and others for whom fertility preservation is an option, as well as the implications for cancer care providers. We will discuss how to recognize psychosocial distress and the type of communication skills that are necessary when counseling patients. Finally, we will describe the ethical implications of fertility preservation techniques and provide resources for the healthcare provider.

## Psychological needs of patients undergoing fertility preservation

The rapidly growing field of fertility preservation has focused primarily on medical aspects of treatment for cancer survivors. Currently, less is understood about the psychological needs of men, women and children who would benefit from fertility preservation. Recently, Tschudin and Bitzer reviewed the literature from 1998 to 2008 [1]. They identified only 24 studies that met their inclusion criteria on the psychological aspects of fertility preservation. Most the current studies on patient's experiences with fertility preservation have explored the issue in the context of sperm banking [1–2]. At present, no studies exist to explore systematically the emotional impact and effects of procedures such as oocyte freezing and ovarian or testicular cryopreservation [1, 3–6]. We do know that fertility preservation is an important issue for cancer patients [1–2, 7–10]. We also know that health professionals, patients and parents consider fertility preservation an important option for young cancer patients [1–2, 10–12]. Nevertheless, all parties involved in cancer care have information deficits and, despite the need for counseling patients about fertility preservation, it is not routinely offered to all patients needing it [1, 2, 8, 13–18].

Individuals diagnosed with cancer during their reproductive life span experience a life crisis in two respects. Firstly, the diagnosis in itself may evoke emotional distress [19, 20]. Secondly, cancer treatments may impair fertility and have profound implications for the patient's quality of life after treatment. We know from investigations of infertility in non-cancer patients that infertility in itself can be associated with grief and depression, increased anxiety [21] and reduced life satisfaction [22–24]. Some patients may experience infertility with the same emotional distress as is typical of an AIDS diagnosis [25]. Although cancer survivors may be able to become parents though egg and embryo donation, gestational surrogacy or adoption, these paths to parenthood may not be available or may be prohibitively expensive. In the case of adoption, survivors may face discrimination in their efforts to adopt, particularly with international adoption, because of their history of cancer [26]. Many countries, for instance, China, will not place a child with an individual or family who has had a history of

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any type of cancer, regardless of stage or years since treatment [26]. Being deprived of the tasks of parenting can evoke profound feelings of loss [27, 28]. Thus, the cancer care team may face an individual who is highly distressed and the team must convey complex, life-impacting information to an individual who has many treatment decisions to make.

## Women

A young woman with breast cancer must time her appointments with a fertility specialist to fit with her menstrual cycle and is often seen by her reproductive physician between surgery and chemotherapy. She may have feelings about surgery in itself, fear losing her life and dread losing her hair or suffer other worries about her body. She may experience the pain of reduced self-esteem, decreased sexual feelings and she may fear rejection from her current or future partner [29–31]. She may have had difficulty locating a reproductive endocrinologist. She must find the funds to pay for fertility treatment along with the money needed for cancer care, and she has no guarantee that the fertility-sparing treatments will work. A young woman without a partner may have to decide between freezing eggs or embryos. If she chooses embryos, there is the additional issue of choosing a sperm donor and, if she has a boyfriend, the complexity of anticipating the impact of her choices on her current and future life. She may not want to pick the father of her future children during this stressful time. She may worry about how pregnancy will affect her life (e.g. if she has hormonally sensitive cancer) or the health of her children. Should she see a genetic counselor? She may feel isolated and alone, not part of the reproductive community and too young for such a serious illness. If single, she may worry about discussing her fertility status with a desirable mate, fearing rejection from her current or future boyfriend and his family [29]. Cancer diagnosed during a young woman's reproductive life may occur just when she has worries about ever finding "Mr. Right" accompanied by questions such as "What's wrong with me?" Potential loss of fertility can add to her already shaky self-esteem and worries about her desirability as a mate. Friends and relatives may try to reassure with platitudes, "Just be glad you're alive" or "You can always adopt," not understanding the emotional complexity she is suffering. If she has children, she may have work or have child-care pressures and may find the many medical appointments to be a burden. She may worry that she is not able to take care of her family as she would wish. For some women, the possibility of not being able to complete her desired family size hurts, and comments to the effect that "You should be glad you already have a child" are very painful. Although cancer usually brings couples closer, cancer can occur during a stressful time in a couple's life. If a couple is in the midst of a stressful period, they must decide whether or not to freeze oocytes or embryos or both, and will this decision exacerbate their stress?

## Men

In addition to anxiety and distress about cancer itself [19], male cancer patients may worry about how treatment will affect their masculinity, role, identity and sexuality [29, 32]. If the patient anticipates role changes because of cancer treatment (e.g. his partner must take care of him) these changes in domestic arrangements may affect how the patient feels about himself and may exacerbate pre-existing couple conflict as well as negatively impacting on sexuality. Younger couples may find it particularly difficult to adapt to new domestic roles and to experiencing the many life and financial issues associated with medical treatment [29]. Even under the best of times, some couples find it difficult to discuss their intimate lives with each other, and may find it especially challenging to discuss these concerns with their physicians and medical staff. If treatment will cause infertility, many men worry about the impact of infertility on their present and future lives and fear for their children's health [29].

## Adolescents

Adolescence is challenging time to help young men and women make appropriate decisions regarding fertility preservation and sperm or oocyte freezing. The caregiver must be sensitive to the impact of the cancer on the patient. An adolescent may be concerned about his or her body, appearance and sexuality [29]. In addition, this is a period where relationships with peers and parents are changing and identity formation is occurring. Cancer may affect the patient's sense of value of him- or herself [28, 29]. Adolescents may be pessimistic about their future, disillusioned with parenthood and afraid of transmitting cancer to their offspring [28, 29]. Balancing parental desires and the authentic needs of the patient is a challenge. Is the patient feeling embarrassed, afraid of failure, rejecting

authority, etc., if he or she declines to freeze? Are the patient's parents unduly influencing decision making? Will the patient's desires change in the future? In addition, information appropriate for the adolescent patient may be lacking. Chapple *et al*. describe the embarrassment and difficulty of discussions of sperm storage with young men and their criticism of sperm banking facilities [33].

## Children

The challenges associated with fertility preservation are even more pronounced with children. We know parents usually want to preserve fertility for their children. Van Den Berg *et al*. interviewed the parents of 318 boys at the time of diagnosis and 2 years later [34]. They found that 61% of the parents would approve of spermatogonial stem cell cryopreservation collected at time of diagnosis. However, children may be too young to comprehend the fertility implications of their cancer treatment and parents may want to shelter their children from such discussions [35]. In addition, parents may not know how to find specialized centers working with ethics board-approved research protocols for fertility preservation involving tissue cryopreservation for children. Presently, it is difficult for the caregiver to counsel parents about the future efficacy of such experimental procedures.

## Lesbian, gay, bisexual and transgender

Evidence based information addressing the emotional and medical needs of gay and lesbian patients is lacking [36]. There is even less research on fertility preservation in the lesbian, gay, bisexual and transgender (LGBT) community. The American Cancer Society reports that approximately 7.5% of the US population is gay or lesbian. Assuming similar rates of cancer in LGBT patients, thousands of patients need counseling about their family-building options. There are many barriers to culturally competent quality health care. Some of the barriers include lack of insurance policies covering unmarried partners, fear of or actual past experience of discrimination by healthcare providers. Anticipated or actual experiences with discrimination may cause some men and women to ignore recommended screenings for breast, colon and prostate cancer, thus preventing early detection of cancers [37]. Not every fertility program will work with a gay person or couple. Many have policies that they will only work with heterosexual, married couples.

# Patients need timely, accurate information

We know that the desire for biological parenthood and genetically related children is important to cancer survivors [1, 2, 7–9, 11, 35, 38–41]. Green *et al*., using a series of semi-structured interviews with 15 young male cancer patients, reported that all of his young subjects found the possibility of infertility disturbing [39]. Zebrack *et al*. found that 59% of a convenience sample of 32 childhood cancer survivors between 19–37 years old, who were interviewed 5 years beyond diagnosis, reported being uncertain about their fertility status despite the fact that all of the cancer survivors expressed a desire to have children in the future and judged parenting and family as very important [35]. The percentage of patients with concerns about their future fertility differ somewhat in the various surveys. Schover *et al*. found 32% were concerned in a sample of male patients from a tumor registry [10]. Zanagnolo *et al*. found that 57% of patients with ovarian cancer were concerned [40]; Partridge *et al*. found 57% of breast cancer patients were concerned [8] and Saito *et al*. found 60% of male cancer patients were concerned even though they had banked sperm [41]. Many surveys of cancer survivors have found that patients are at risk for emotional distress if they become infertile due to cancer treatment [e.g. 1–3, 5–7]. Patients (both men and women) who have not had children prior to their medical diagnosis are more distressed about infertility than those who have already had their children [7, 8, 11]. The desire to have children may influence the type of treatment a patient chooses [8] and may help the patient emotionally cope with their cancer [41].

Yet despite the fact that patients may be very upset about loss of fertility, there are many problems with timely and effective communication between cancer care specialists and their patients. The most significant barrier to fertility preservation is the fact that many care providers simply do not convey information about fertility-sparing options to their patients [1, 2, 8, 14–17, 42, 43]. Duffy *et al*. found that only 34% of 166 premenopausal women recalled discussions about fertility preservation before beginning chemotherapy with any of their healthcare providers [15]. Thewes *et al*. reported that 71% recalled discussing fertility-related issues with their physicians, but that 45% said that they initiated the discussion themselves [16]. Similarly, Goodwin *et al*. found that parents and patients

initiated discussions about fertility preservation themselves [44]. Partridge *et al.* found that 26% of women in a web-based survey of 657 young breast cancer patients felt that their concerns about fertility and reproduction had not been adequately addressed at the time of diagnosis [8]. Oosterhuis *et al.* surveyed 97 parents of pediatric patients and 37 adolescent patients and found that only 29% of the parents and adolescent patients were satisfied with the information they received about fertility-sparing options [42].

There is a debate about the meaning of "lack of recall" about having been provided information (or not) about fertility preservation, with evidence on each side of the debate. Most simply, a lack of recall of any discussion may indicate that information was not provided. In Duffy *et al.*'s investigation, 34% recalled discussion about fertility-sparing options and 100% of the patients recalled discussions about the impact of chemotherapy on their everyday lives [15]. If the difference in recall is due to information given (but not recalled), why do 100% of the patients recall discussing the effects of chemotherapy on their everyday lives? On the other side of the debate, Van Den Berg *et al.* investigated 202 parents of 117 male childhood cancer patients who had been informed about fertility during consent [43]. Only 50% recalled statements about fertility and 36% denied receiving any information [43].

We know that anxiety and emotional distress can impair the ability to register information [45]. We also know that anxiety can inhibit information processing [46, 47]. In addition, lack of information may lead to emotional distress [48–50]. Thus, a patient's lack of recall may be due to many factors, not simply due to provider omission. Nevertheless, we know that there are challenges to effective communication between cancer care specialists and their patients. Quinn *et al.* found that the physicians in their investigation usually discuss fertility loss as a side effect of treatment for cancer [14, 51]. However, few actually provided specific information for their patients to preserve their fertility. Reasons cited by Quinn *et al.* [14, 51] and others [e.g. 8, 17, 52] include a lack of knowledge of options for fertility preservation, a challenging medical setting, a lack of time for the discussions and a lack of training in how to discuss fertility issues [17]. Providers also indicated that gender and cancer site as well as the costs of fertility preservation procedures and access to the necessary resources were factors that influenced their decisions regarding discussion about the full range of options available [14, 51, 52].

At this point, there is general consensus that fertility preservation is very important to patients and that patients affected by cancer during or prior to their reproductive life span should be informed about possible fertility impairment due to cancer treatment [53–57]. Adult patients, parents of minors and (if possible) minors should be provided up-to-date realistic expectations about the success rates of fertility preservation, the cost of these procedures and referral to appropriate facilities. Parents of minors should be referred to appropriate specialized research centers. The discussion of fertility-sparing options should address common patient concerns about whether or not fertility preservation will decrease successful cancer treatment or be harmful to the patient or to the patient's offspring, short and long-term side effects of treatment on the reproductive system, pregnancy risks and concerns about the possibility of genetic risks to the offspring. In addition, physicians and the cancer team should discuss the option of deciding not to preserve fertility.

We know that physician recommendations about fertility preservation are very influential. Schover *et al.* found that physicians' encouragement to bank sperm was almost as strong a predictor of whether or not a patient banks sperm as the patients' own desire for future children [10]. Schover *et al.* noted that the influence of provider recommendations is consistent with what is known about the effectiveness of physician recommendations about smoking and early cancer screening [10]. Saito *et al.* found results consistent with Schover *et al.*'s findings [41]. They interviewed 25 cancer survivors who had banked sperm at their own initiative and 26 patients who had banked sperm with their physician's recommendation. Patients who banked sperm with their physicians' instructions felt better about having cyropreserved sperm. These results underscore the importance of the cancer care team's communication and encouragement in exploring options to preserve the capacity for biological parenthood.

## Provider communication skills and communication pitfalls

Information empowers patients to ask pertinent questions and may avoid emotional distress by providing options and realistic expectations. Lack of information can lead to emotional distress [48, 58, 59]. In addition, poor communication skills can profoundly influence the quality of life of patients. Kerr *et al.* prospectively

investigated the impact of patient-rated provider communication skills with 990 cancer patients [60]. They found that 45% of these patients reported that some aspect of communication with their physicians was unclear and 59% of the patients wanted to speak to their medical team more frequently. Age was a factor in patient satisfaction. Patients under 50 years of age rated social and psychological help as more important than medical information. Patients who reported that information was incomprehensible or incomplete described significantly worse quality of life on 17 variables up to 4 years after diagnosis.

A number of studies have found that effective communication significantly impacts patients' satisfaction and quality of life [61–70]. For instance, Roberts *et al.* investigated the types of communication cancer patients find helpful [71]. They found that the best communication and psychological adjustment occurred when physicians had a caring attitude, demonstrated empathy and spent sufficient time with their patients. Patients found it very important that their physician engaged them in collaborative decision-making [71]. Common communication pitfalls are lecturing, stopping the patients from addressing their own concerns, depending on routine procedures and discussions and premature reassurance. Back *et al.* and others recommend assessing a patient's pre-existing knowledge and questions, providing information in small units, followed by understanding the patient's comprehension and evaluation of the information provided [72].

Thus, while discussions of response to treatment and the types and severity of side effects is important, patients also need attention to their coping and psychological well-being. They should be encouraged to speak about the complex psychosocial issues they are facing because of the cancer diagnosis and fertility treatments. Addressing these psychosocial issues is important for achieving treatment goals and engaging patients in their own care. Patients should be encouraged to bring trusted friends and family to provide emotional support during and after the visit. Given the anxiety and potential inability to register information, friends and family can also help to process information and, if necessary, ask relevant questions. Patients should also be provided a list of clearly written instructions and resources to help reinforce the information given during an office visit and enhance treatment adherence. Because of the time-sensitive nature of fertility treatments, patients should be helped in finding fertility specialists who are familiar with the need to "fast-track" cancer patients.

## Emotional distress associated with cancer

The prevalence rate of clinically significant emotional distress (depression and anxiety) in cancer outpatients varies considerably from study to study. Strong *et al.* reviewed studies of clinically significant emotional distress of cancer patients and reports prevalence rates from 15 to 42% [73]. The majority of studies have been small, used different measures of depression and anxiety and had different sample characteristics. Strong *et al.* investigated 3071 cancer outpatients with a variety of cancer types and found that nearly one quarter had significant emotional distress [19]. Being young, female and having active disease were independent predictors of emotional distress.

Costanzo *et al.* wished to clarify the difference in reported prevalence of psychological distress in cancer patients by conducting a longitudinal study of 206 patients with age, education, gender and demographically matched controls [74]. They found that survivors, as compared with the matched controls with no cancer history, had worse psychological functioning in a variety of areas including mental health, mood, environmental mastery and self-acceptance. Their investigation also showed that these differences existed prior to diagnosis in the individuals who later developed cancer. They suggest that the cancer diagnosis exacerbated prior psychological difficulties and that these difficulties may actually put individuals at risk for developing cancer. They are not suggesting that depression and anxiety cause cancer. Rather, they hypothesize that psychological distress may be related to poor health behaviors such as smoking, poor diet and lack of physical exercise and that these behaviors put the individual at risk for developing cancer.

Costanzo *et al.*'s study is important in that it was a prospective investigation with matched controls. However, this evidence must be weighed against another prospective study of the prevalence of emotional distress in a normal population. In a longitudinal prospective study of depression, anxiety disorders, alcohol dependence and cannabis dependence, Moffitt *et al.* report that nearly 60% of the population experiences at least one of these mental disorders by age 32 [75]. In their investigation Moffit *et al.* assessed more than 1000 New Zealanders for mental disorders

11 times between ages 3 and 32. Their study focused most intensively on the period from age 18 to 32. Most investigations of the prevalence of emotional distress in the USA and New Zealand rely on retrospective self-reporting. Self-reporting may underestimate emotional distress because it relies on individuals' ability to remember and their willingness to disclose their past experiences. On the other hand, it may also inflate prevalence rates because researchers may assign mental ailments to people with mild symptoms of no real concern.

Thus, psychological distress may precede, exacerbate or follow a cancer diagnosis. More research needs to be done to clarify these complex interactions. However, we may safely say that many cancer patients suffer significant emotional distress. The breast cancer literature suggests that younger women suffer more anxiety and depression than older women. Young women typically report more emotional distress, more unmet needs and worry about finances. They also report more loss of time at work, more child-care problems and decreased self-image when compared with older women [76–88]. A young woman's stage of life may create multiple stressors that interact with her cancer diagnosis leading to increased psychological distress [87, 89–91]. Young women often experience psychological distress with the loss of menses during chemotherapy. The loss may cause young women to feel older and experience themselves as different from other women their age. They may feel vulnerable and have concerns about the potential loss of fertility [38, 92]. Even women who have completed their families suffer from the loss of choice to have more children. However, psychological distress is more significantly related to infertility in women who have not yet started their families and would like to do so [89, 92, 93].

Sexuality is often affected by chemotherapy-induced menopause. Younger women receiving chemotherapy are at risk for lower levels of sexual functioning, assessed by decreased libido, difficulty reaching orgasm, vaginal dryness and reduced sexual satisfaction. Younger women are more likely to view themselves as less sexually attractive than they did before therapy [94–100].

Less research has been conducted on the psychosocial needs of men as compared to women [101]. Kiss and Meryn reviewed studies of prostate and breast cancers to compare the psychosocial effects of cancer on men and women and to compare their reactions [102]. They found that the diagnosis of cancer is distressing for both men and women. Cancer site is less important than disease stage, pain and absence of social support [101, 102]. Both men and women benefit from social support, though men are more comfortable turning to their partner. Women often seek support from a larger network of people.

Ream *et al.* investigated 1848 men who had been diagnosed with prostate cancer during the previous 3–24 months [103]. In this postal survey, almost one third of the men reported extreme anxiety or depression. The men also reported unmet needs for social support. The men wanted help for their emotional distress, sexuality-related problems and treatment side effects. The young men reported more unmet needs about their sexuality, especially if their treatment involved a radical prostatectomy.

More research needs to be done on the impact of cancer on men's gender identity and sense of masculinity, particularly the interaction of such factors as stage of life, sociocultural background and fertility status. Green *et al.* investigated the psychological reactions of young men who were infertile due to cancer treatment [104]. The young men described intense reactions such as depression and anger about their cancer diagnosis and treatment. Younger men who had not known that their ability to parent a biological child would be affected and who wished to have a child reported the most powerful reactions. They were angry that they had been denied a highly significant life choice; expressed rage at the injustice of human suffering; and were irritated they had not been informed about the side effect of infertility.

## Predictors of psychosocial distress

Green *et al.* studied 160 stage I and stage II node-negative breast cancer patients to assess psychological and demographical predictors of psychological distress [105]. Women with past histories of trauma, previous psychological distress or those experiencing additional stressful events, are most at risk. Younger women with children at home are most vulnerable to psychological suffering. The investigators recommend that the cancer team assess psychological risk by collecting information about the women's histories. Since cancer can trigger painful unresolved emotions, the cancer team needs to address prior trauma such as sexual abuse. For some women, the invasive nature of the procedures and the loss of control of their bodies can re-traumatize them [106–113].

There has been extensive investigation of coping styles in cancer patients [114, 115]. Some coping styles are associated with less emotional distress and better psychological adaptation to cancer. Adopting an internal or personal locus-of-control, being optimistic and taking a minimizing perspective are associated with reduced emotional distress. In addition, problem-solving, having a "fighting spirit", positively reinterpreting problems and seeking social support, are other helpful strategies for dealing with cancer. In contrast, avoidance or escapism is associated with more psychological distress. Behaviors such as wishful thinking, blaming oneself and adopting a resigned attitude are associated with more emotional distress. Stanton *et al*. studied 92 breast cancer patients and found that actively processing and expressing emotions enhanced physical and emotional adjustment over a 3 month period [116].

The cancer team can provide better care by understanding the emotional needs, psychosocial predictors of distress and methods of coping that benefit cancer patients. It is important to note that trying to change a patient's style of coping by encouraging them to adopt a "fighting spirit" or "stop being fatalistic" is not as helpful as an empathic stance tailored to the emotional needs of the individual patient [117].

## Cross-border reproductive care

Although the first recorded instance of cross-border reproductive care (CBRC) occurred in ancient Greece over 2500 years ago, the phenomenon is rapidly growing [118]. Patients travel for a variety of reasons, including technology advances; affordability; rapid access to care; procedures not widely available; privacy concerns; and improved standards of care. The cancer team needs to know common patient concerns, whether or not they are a travel or destination country. Patients need trusted sources of information; information on treatment options, risks and cost; international accredited clinics; and counseling to understand the psychosocial dimensions of their choices. Providers also need trusted information, as well as data, on costs and outcomes, standards of care and protocols for advising patients on CBRC. The cancer team may be asked such questions as, "Where can I go to preserve my fertility?" "Is it safe?" "Is the facility accredited and by whom?" "Are the caregivers adequately trained and certified?", etc. Patients need adequate counseling before departure, especially if gamete donation or surrogacy is involved. They need information and counseling in the destination country that is adequate and provided in a language that is understandable to the patient. Legal issues may be complex when traveling between countries (or even within countries). For instance, within the USA gestational surrogacy is not legal in some states. Referral to a lawyer experienced in the issues in both sending and receiving countries is very advisable.

## Conclusion

Fertility preservation is a complex field involving many specialists from different disciplines. Psychologists or other mental health providers should be included in a team approach to fertility preservation. Many cancer patients would benefit from referral to a mental health provider for additional attention and support. Mental health providers can counsel patients about the complex decisions they are facing when considering fertility preservation. Patients may feel more at ease discussing mood changes, sexual difficulties, self-esteem problems and other life stressors with mental health providers than with their oncology physician or surgeon. Psychologists and social workers may have training, or know how to get information, about CBRC and be aware of the issues involved in cultural competency. Thus, a team approach allows each care provider the ability to stay within their professional scope of practice. Most importantly, when the emotional needs of patients are addressed, normalized and considered standard of care, cancer patients may feel understood and most fully cared for.

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Chapter 41

# Fertility preservation

## Ethical considerations

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## Fertility preservation and ethical considerations for adults and children

Fertility preservation is a new subfield of reproductive medicine aimed at preserving the potential for genetic parenthood in adults or children at risk of sterility before undergoing anti-cancer treatments. Modern and powerful chemo- and radiotherapy protocols are either curing or significantly extending the survival for many young patients with cancer. Five-year survival rates for Caucasian and Hispanic American women have increased for Hodgkin's lymphoma from 86 to 98% in the quarter century before the year 2000 and for breast cancer from 78 to 91% [1]. At the same time, diagnoses of some malignant diseases have become more prevalent (e.g. breast and testicular cancer) [2]. The net effect has been an increase in numbers of patients in the reproductive age window (and younger) at risk of sterilization or early menopause by the effects of ionizing radiation or alkylating agents such as cyclophosphamide and platinum-based drugs [1].

As a result of this progress, quality of life issues after cancer are emerging. Included in this quality of life rubric is the possibility of protecting fertility from the toxicity of these efficacious but noxious treatments.

Many strategies have been devised to pursue fertility preservation (Table 41.1) [3]. Embryo freezing is well established but it is not always an applicable option; for example, for women who are not married, for women requiring immediate treatment or for young pre-pubertal girls. Others techniques like oocyte freezing, ovarian tissue or whole ovary freezing are still considered experimental. Likewise for men, the option of semen cryopreservation before chemo/radiotherapy is well established. Spermatogonial harvesting and testicular tissue freezing for later transplantation or even xenografting are highly experimental.

**Table 41.1** Established and experimental options for preserving fertility

| |
|---|
| **Established** |
| Ovarian translocation (oophoropexy) to avoid a radiation field |
| Radical trachelectomy for cervical cancer |
| Cryopreservation of spermatozoa |
| Cryopreservation of embryos |
| **Experimental** |
| Cryopreservation of oocytes |
| Cryopreservation of ovarian tissue |
| Cryopreservation of whole ovary |
| Folliculogenesis in vitro |
| In-vitro oocyte maturation |
| Cryopreservation testicular tissue |
| Cryopreservation spermatogonial cells |

In using both therapeutic and experimental techniques informed consent is essential. In presenting the option of therapies, women and men have the right to know their options concerning fertility preservation and the risks and costs involved. Consent to therapy may require involving a surrogate decision maker in the case of young children or mentally impaired persons. Providing thorough informed consent in recruiting persons to participate in research is the foundation of the ethical conduct of research. It is based on three components: adequate, comprehensible information; a competent decision-maker; and a voluntary decision process. Research should also be reviewed and approved by Institutional Review Boards. Patients have the right to know what will happen if they or

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any children that are created are injured or disabled in terms of health insurance and compensation. Unfortunately, much of the existing literature on informed consent using reproductive technology has focused on information disclosure with an eye toward minimizing professional liability. Those involved in using experimental techniques must focus not simply on disclosure but on comprehension. The use of quizzes and documenting responses to questions after information is presented, are effective tools to assist in documenting that patients understand the experimental or innovative nature of some modes of fertility preservation.

## Adults

From an ethical standpoint, the key reason for pursuing fertility protection is to restore personal autonomy to those who are unable to conceive [4]. However, since many of the technologies are innovative but yet highly experimental, it is difficult to design clinical trials: how to provide a proper informed consent and insure respect for autonomy. Who to include or exclude in trials of innovative techniques and how best to recruit them? The presentation of risk information is complicated by the fact that both the adult and their offspring may be involved. A core principle of medical ethics is to do no harm. If ovarian tissue or testicular tissue cryopreservation is to be tested, then the level of risk that can be tolerated should require essential careful animal studies and close oversight of research by review committees. It is reasonable in the absence of grant funds to seek reimbursement from patients to cover the expenses of the research, but there should be no charge for clinical fees until the experimental options have been proved safe and effective.

Ideally the decision about who is candidate for fertility preservation should be rendered by a team including a medical oncologist, reproductive endocrinologist, a pathologist and a psychologist, all guided by written protocols which can be shared with patients [4]. Patients should not be provided with false hopes, and alternative plans including no intervention with the prospect of adoption or childlessness should also be part of the discussion. Equity or ownership interests in novel technologies utilized in research must be disclosed to potential subjects.

## Children

Impaired future fertility is another possible consequence of exposure to cancer therapies even for children. This risk may be difficult for children to conceptualize, but potentially traumatic to them as adults. Unfortunately, the modalities that are available to children to preserve their fertility are limited by their sexual immaturity and are essentially experimental.

For boys who cannot produce mature sperm, harvesting and cryopreservation of testicular stem cells with the hope of future autologous transplantation or in vitro maturation represents potential methods of fertility preservation. For girls, isolation and cryopreservation of ovarian cortical strips/primordial follicles followed by in vitro maturation of gametes when fertility is desired is a possible option. Extensive research is still required to refine these modalities in order to safely offer them to patients as therapies [4]. Assisted reproductive technologies (ARTs) must be scrutinized on the basis of efficacy and safety and they must be subjected to rigorous ethical deliberation by independent review committees before they can be offered. The modalities involved in fertility preservation of young children are no exception to these rules. In addition to ensuring that the basis for offering the intervention is scientifically sound, the execution of the intervention must be deemed ethically sound. This determination requires that the intervention in question be evaluated within an ethical framework that considers it in terms of beneficence, respect for persons (autonomy) and justice [5]. It can be argued that fertility preservation aimed at children is ethical because it prevents morbidity (reproductive and psychosocial) and it safeguards their reproductive autonomy [6]. Therefore, the main ethical question concerns the process involved in achieving fertility preservation and the techniques required. The answer is found in an exploration of the potential risks of the intervention to the patient and his or her progeny, the special situation of children as research subjects and patients, and the potential abuse of the technologies in the future [6, 7]. Programs must make every effort to minimize financial barriers to access for children and to work with patient advocacy groups to seek coverage for children and families who cannot afford to participate in fertility treatment or research.

Children represent a unique and vulnerable population with respect to medical research. They have diminished autonomy, diminished capacity to understand risks and benefits of research objectives and lack the ability to provide consent for research studies. As a result, they require special protection against potential

violation of their rights that may occur during research investigations [5, 8]. Until very recently, institutional attitudes impeded significant participation by children in medical research for fear of exploitation [5]. This attitude was attributed to several historical episodes of the unethical targeting of children as medical research subjects. Ethical guidelines to protect children as research subjects were outlined with the publication of the Belmont report in 1979, generated by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research. Children should not be exploited to participate in pediatric research, nor should they be deprived of the benefits research has to offer because of their vulnerable status. Research involved in childhood fertility preservation should be conducted on patients who could experience personal benefit from the research, eliminating the prospect of exploitation for the gain of others [4].

With respect to childhood fertility preservation, proper attainment of informed consent from a legally authorized representative (i.e. parent or guardian) and of childhood assent must be ensured [5, 8]. Assent – the active affirmation by the research subject – can be obtained from incompetent minors and it should be obtained from children whenever possible. While the benefits of gamete cryopreservation are promising they are largely unquantifiable because human data on the survival of gametes after the freeze–thaw–transplant process is limited. Until more data becomes available we cannot tell patients what percentage of gametes will survive and what the probability of conception is, and we must not provide them with false hope. Alternatives to gamete cryopreservation should be discussed and patients should be given the option of no intervention [6]. Barriers to the consent process for fertility preservation interventions may develop. While parents may be competent to consent for their children, the scenario is very complex clinically and emotionally. It has been suggested that to overcome some of the practical obstacles involved in the consent process, it should be performed in stages [9, 10]. If a two-stage process is adopted, the issues of gonadal harvesting/storage and gamete manipulation can be handled as two separate topics at distinct time points. The decision to harvest gametes would be made at the time of cancer diagnosis and consent for the procedure would be left to parents/guardians. The decision of whether to use the gametes after they have been isolated can then be made at a future point by the child when they reach adulthood. At such a point in time, the young patient would be better able to express personal preferences about the handling of the tissue based on an enhance capacity to understand the ramifications of the possible medical interventions available at that time.

## Sterility after cancer and use of donor gametes

Cancer survivors who did not preserve fertility and became sterile after chemo- or radiotherapy may agree to the use of donor gametes (oocytes or spermatozoa). The pre-eminent ethical issues here are: (1) Should couples resorting to the use of donor gametes be obliged to disclose such use to their children once they reach the age of understanding [11, 12]? (2) Should the utilization of donor gametes follow policies analogous to those governing disclosure to children that are adopted [13]? (3) What will be the welfare of a child if he or she "finds out" that a gamete donor participated in his or her creation and this was kept as a secret? (4) Should a woman's age and life expectancy factor into a clinic policy concerning access to services? (5) How hard should a clinic try to establish what parenting arrangements have been made in the event of the death (or cancer recurrence) of the would-be mother or father, since little is known about the capacity for post-cancer women to parent infants and toddlers?

Without clear guidelines the ethical propriety of the technologically driven options for parenting after cancer (with donor gametes), becomes a matter of the marketplace.

Mandating disclosure by a couple is problematic on a number of fronts and the claim that the practice of gamete donation is similar to adoption is not obvious [13]. With gamete donation, one of the parents is the biological parent, while the other is the social parent; with adoption, both parents are social parents. With gamete donation the child's mother, whether biological or not, carries the pregnancy with all of the accompanying psychological interplay. In the case of a donor egg, to ask such a mother (and she is a mother under any definition of motherhood) to tell the child that technically she is not the mother because she is not genetically related may strain the bond between mother and child [13].

Certainly the pre-eminent concern is the welfare of the child [14]. However, the benefits accruing to a child from disclosure are difficult to assess and still

remain open to many interpretations. In debates about child welfare with adoptions, researchers and policy makers have not yet provided a clear consensus on what is the best interest of the child [14]. In addition to considerations of child welfare, one must address the impact on the other participants in the process, including the donor, the couple and the healthcare providers. A donor has to be willing to donate his sperm or her oocytes knowing that later he or she can be identified; the couple has to agree in revealing the origin of the gametes to their offspring; the providers have to restrict reproductive services only to couples who agree in writing to disclosure. Without uniform requirements for the collection and maintenance of records, the usefulness of revealing information is not clear.

To force fertility specialists to participate in implementing disclosure is ethically problematic as well. Including acquiescence to disclosure as part of screening criteria or recruitment to protocols may unjustly interject social factors into criteria for program acceptance. It may also encourage desperate couples to lie if they disagree. Since relationships vary greatly with background, ethnic origin, and attitudes of both the male and female partner, it would not be unreasonable for a healthcare provider to raise the issue of informing offspring of his or her genetic background at the time the use of donor gametes is considered by a couple. To do more than to raise the issue and present known data on the potential psychological and health ramifications of disclosure and non-disclosure, could violate the privacy of the couple's relationship and interfere with their ability to decide. Programs that use donor gametes should, however, prepare policies on how to handle in future requests of information from children of donor gametes. They may choose to disclose only non-identifying information when the requesting party has reached 18 years of age, or they may disclose more based on the particular willingness of donors to remove more of their information from anonymity [15].

In countries where the identification of the donor is required by law, such as the Netherlands, Sweden and the UK, the pool of sperm donors has substantially decreased and many clinics have ceased using this option. Despite this concern, legislation in the UK enacted in April 2005 imposed mandatory disclosure for donor sperm. As a result of donor's unwillingness to be identified, there has been a marked reduction in the frequency of donor inseminations. A BBC survey in September 2006 found that 90% of UK sperm donors were recruited in just 10 of 87 licensed clinics for donor insemination and that after the removal of donor anonymity, the cost of purchasing sperm rose very substantially; about an eightfold increase. Between 2004 and 2006 there was a 30% reduction in patients requiring donor insemination but a much larger, about 45%, reduction in the number of treatment cycles using donor sperm (http://www.geneticsandsociety.org/search).

## Pre-implantation genetic diagnosis for designer babies

The ethical ramifications of pre-implantation genetic diagnosis (PGD) are essentially related to the issue of requesting embryo biopsy for non-medical indications such as family balancing (also known as gender selection or preconception sex selection) and HLA-matching (also known as designer babies). Simply stated, is it permissible to select embryos based on HLA compatibility? And if not, what are the moral justifications to deny it? Is it morally justified to create embryos and then transfer only the ones HLA-matched with an existing sibling, so to design a perfect tissue-donor? The medical reasons clearly offset the moral arguments of gender selection for the prevention of gender-specific genetic disorders (e.g. Hemophilia [XLR], muscular dystrophy or incontinentia pigmenti [XLD]). But in the absence of clear medical indications – like PGS for family balancing – the moral arguments to deny this service become stronger. Using procreation and reproductive technologies as a means to save another sibling life could be seen as exploitation. Creating a child (savior) whose own value and identity could later be affected by the act of being an organ donor for their sibling requires a full psychological evaluation of the requesting families and the risks of instrumentation and exploitation, albeit potential, need to be fully addressed.

Currently, only a minority of couples seems to give importance to the sex of their children and even less seem to be willing to use the service of preconception sex selection for non-medical reasons [16]. It is important however to establish ethical guidelines and to address, for both HLA-matching and for gender selection requests, the fates of the non-HLA matched embryos and of the embryos of the undesired gender.

## Ethical aspects of ovarian cryopreservation and re-transplantation

Ovarian tissue cryopreservation, thawing and transplantation, either as heterotopic or orthotopic allografts, has shown some reproductive success [17–22]. In addition, many births have also been reported by using fresh ovarian transplants between monozygotic twins [23]. Taken together these reports of pregnancies and birth of healthy children (10 from the use of cryopreserved–thawed ovarian tissue at the time of this writing), bode well for fertility preservation. There is also the report of a birth from the transplant of a whole fresh ovary between two sisters HLA-compatible [24]. However encouraging, these are preliminary results and the technique requires more follow-up.

The time is now for ethical considerations of ovarian cryopreservation and re-transplantation compared to the other two options of oocyte and embryo cryopreservation.

Preserving ovarian tissue may result in less intervention both before and after re-transplantation (the ovarian tissue could contain thousands of oocytes) and can create more opportunities to conceive than oocyte cryopreservation. Ovarian tissue harvesting does not require cycles of hormonal stimulation as is needed with oocyte extraction. Furthermore, once the ovary is re-transplanted, pregnancies can follow within a woman's own natural endocrine cycle without the need of in vitro fertilization (IVF). In contrast, with oocyte cryopreservation each cycle of ovarian stimulation usually yields 10–15 oocytes, thus to "bank" an adequate number of oocytes (to ensure likelihood of a successful pregnancy in the future), multiple hormonal interventions are required.

Lastly, faith traditions may favor ovarian tissue transplantation over IVF because it is simply a reintroduction of the women's own ovarian tissue and permits "natural" conception in vivo. This procedure may therefore hold great promise for women whose faith traditions prohibit other fertility treatment methods, though a thorough evaluation of reception of ovarian cryopreservation by faith traditions has yet to be performed.

Some disadvantages of ovarian cryopreservation and re-transplantation, when compared to oocyte cryopreservation and embryo cryopreservation, can be anticipated. First, there is no opportunity to perform PGD without further intervention. Embryo and oocyte cryopreservation can both provide an inherent option for PGD. As identifiable genes for diseases and phenotypic traits continue to be uncovered, the option of PGD may become increasingly desirable. Though IVF could be performed and PGD could be done post-transplantation of ovarian tissue, this would require an extra step and an extra invasive procedure.

The option of "designing" one's child may overshadow the positive aspects of ovarian cryopreservation and re-transplantation, especially if there is a family history of genetically transmitted disease. Ovarian cryopreservation and re-transplantation could be considered unpredictable regarding which traits the child inherits.

Second, ovarian tissue cryopreservation and re-transplantation presents a multifaceted potential for false hope. False hope in medicine can be defined as "based on a set of unrealistic expectations, encouraged through incomplete or faulty information or by a patient's unwillingness to acknowledge the limits of medicine" [25]. Currently, there is no guarantee that ovarian cryopreservation and re-transplantation will result in future offspring, just as in embryo and oocyte cryopreservation. A reasonable success rate should be established prior to the mainstream offering of this technique as non-experimental. The procedure should only be offered with a clear statement of risks and benefits [26]. As this technology is further developed, a romanticized view of forever preserving fertility is imaginable, which may place unrealistic expectations on this technology. This reality must be clearly indicated in order to avoid major disappointments. Though any new procedure has the potential for false hope, it is important to acknowledge the limitations of the procedure to the best of one's ability, especially when the hope is for the creation of a new life.

## Future: stem cell research and the manufacturing of gametes

Cancer survivors that have failed to preserve their gametes prior to sterilizing treatment might benefit in future of a type of stem cell research aimed at the creation in vitro of gametes derived from embryonic stem cells [27].

The process would require the following steps:

(a) Use of a somatic cell's nucleus to be inserted into an ooplast (i.e. an oocyte from which its own nucleus has been removed).
(b) Mechanical activation to induce cell's division and growth to blastocyst stage.
(c) Extraction of the inner cell mass, isolation and in vitro growth of cells destined to form germ lines (sperm or oocytes).

The subject of research on embryos created through IVF presents a variety of ethical and legal issues. The central part of the debate is the moral status of the embryo [27]. This debate is not unique to the twenty-first century scientist or bioethic scholars; in fact it can be traced to Aristotle, who wrote of the ensoulment of the human at a particular stage, as did the pre-Socratic philosopher Heraclites before him. Religious views of conception have been extensively debated in Judeo-Christian and Muslim scholarship dating to the earliest religious texts in those traditions. Views on the moral status of the human embryo normally take one of the following three forms:

1. The human embryo has no intrinsic moral status; it derives its value from others.
2. The human embryo has intrinsic moral status, independent of how others value it.
3. Embryos begin with little or no moral status and continue to achieve more and more status as they develop.

*The position that an embryo has no moral status* can be argued in several different manners. Because the fetus fully depends on the pregnant woman for development, many ethicists believe that it cannot be viewed as an unique entity. The moral concerns expressed by those who hold this position about embryo research are focused on the long-term social implications of embryo research for the status of born persons, particularly those with disabilities. However, it is not held that the destruction of an embryo is inherently morally problematic.

*The position that the fetus has intrinsic moral status* is grounded in the view that a *person* is created at a moment in time that can be linked both to the consummation of an act by those who participate in its creation, and to the physical and legal initiation of that person's participation in the human community. The metaphor most often used to describe the status of the fetus for these purposes is that of *baby*; the ever-increasing presence of the fetus in public and private life has contributed to the view that from the "moment" of conception a person can be identified, independent of the risks that face a person so defined, and regardless of the plain differences between such a person (for example in the case of a frozen embryo) and a person who participates as a baby, child or adult in the institutional life of the community. Given this view of conception and the embryo, the use of an embryo for research purposes is exactly tantamount to the use of any other vulnerable subject in research without consent, research that poses not only a great risk but in many cases has the clearly anticipatable outcome of death for the subject.

The moral issues surrounding embryo research leave the status of the embryo highly contested. The lack of consensus about the status of the embryo and the morality of research has resulted in what might be somewhat contradictory and unclear legal definitions in the USA at the state and federal level. Since it is extremely difficult to define the status of the embryo and the question still remains hotly contested, most of the legislation tries to steer away from making a definitive statement.

The legality of embryo research also varies from country to country. Experimentation on the embryo for the purposes of developing stem cell and other technologies, and for general knowledge, is legal in the UK, Singapore and three Australian states under certain circumstances. In Germany and Italy, embryo research is banned completely. In Switzerland it is highly restricted. In the USA, debates over the legality of embryo research vary from state to state, with California having the most tolerant policy and Louisiana among the most restrictive.

Whatever its religious or scientific underpinnings, the ethical debate surrounding human embryonic stem (hES) cells has recently centered on how the hES cells are derived and on whether or not they should be protected from destruction, much like an adult is [27]. Using leftover IVF embryos for the purposes of hES cell research raises complex questions about the status of the embryo, the value of human life, and whether there should be set limits regarding the interventions into human cells and tissues. Furthermore, questions about adequate informed consent, oversight and regulation also come prominently into play.

Those who support hES cell research argue that an embryonic stem cell, even though it is derived from an embryo, is not itself an embryo and thereby would never continue to develop into a fetus, child and adult. Each stem cell is only a cell that can be triggered to become a specific kind of tissue yet could not be triggered to become an individual. Furthermore, the embryo at the blastocyst stage has not developed any kind of nervous tissue and thus extracting individual stem cells would not be painful for the embryo. Since the embryos used for stem cell research come mostly from the leftover IVF embryos, which would otherwise be discarded, the proponents of stem cell research argue that it is better to use such embryos to find cures for debilitating diseases rather than to discard them, benefiting no one.

One attempt to resolve the debate over stem cell research involved the suggestion that researchers might obtain stem cells from embryos without actually engaging in the destruction of those embryos [28]. (It also was suggested that totipotent cells might be removed from four- or eight-cell pre-implantation embryos destined for PGD [28]).

Another central problem is the permissibility of making embryos specifically for research purposes. There are two different types of embryos used: those classified as "spare" embryos which are left over from unsuccessful in vitro fertilization and those produced specifically for purposes of being tested. Some people have ethical concerns about both of these methods; however, those who support research are more likely to question the ethical nature of the second of these two alternatives.

The argument that it is acceptable to use spare embryos but not to create embryos specifically for that purpose centers on Kant's categorical imperative, specifically the formulation of that imperative that centers on the claim that the ultimate moral wrong is to treat someone as a means to some other end, rather than as an end in him- or herself. Those who do not support the use of embryos for the sole purpose of enhancing research argue that it is morally unacceptable to use embryos for scientific purposes on the grounds that this is a clear use of a person as a means. Some of these same arguments can apply to the use of embryos under any circumstances. In the case of spare embryos, by contrast, many are too old or morphologically inappropriate to be implanted, and thus have no other use; it is thus argued that the use of these for research is not nearly as questionable [28].

## Implications for ART clinics

The processes whereby embryos are created (whether from donor eggs and/or sperm intended for research purposes or as a byproduct of reproductive health care), analyzed, stored, thawed or destroyed, are all processes that require, the technologies, clinical expertise, patient population and institutions of ART. It is thus no surprise that the largest research programs in the field have employed reproductive endocrinologists, biologists, ART psychologists and social workers. Ethical issues related to participation in stem cell research include three key problems.

1. First is the question of whether and under what circumstances patients or research subjects should be allowed to participate in the donation of reproductive materials for stem cell research, particularly where that research involves the creation of embryos for research purposes.
2. Second is the question of whether reproductive clinicians and technologists should be involved in the non-reproductive use of cloning technologies for the creation of nuclear transfer-derived stem cells.
3. Third is whether and when clinicians involved in the derivation of embryonic stem cells should be held responsible for the failure of those cells in clinical trials or therapies using those cells.

At this time, there is no real consensus about any of these issues, although all three issues continue to receive the attention of the ethics boards of professional societies, such as the American Society for Reproductive Medicine (ASRM), and of bioethicists.

## Conclusions

If they have lost their reproductive function, cancer survivors may wish to become parents by using previously stored gametes or gonadal tissue. Fertility preservation serves such a wide range of medicosocial circumstances, some quite unique, that patient care requires an individualized and multidisciplinary approach. In particular, fertility specialists offering fertility preservation options to cancer patients should be properly trained and knowledgeable to discuss patient's treatment plan, prognosis, as well as unusual health risks for future offspring and the potential harmful effects of pregnancy.

Overall there should be no ethical objections to offer these services since they are offered with the aim of preserving future fertility.

In practice, however, there are objections:

1. The options available, except sperm storage and embryo cryopreservation, are all experimental. There is lack of extended follow-up about their safety.
2. Posthumous use of stored tissue or gametes. When gametes or tissue is stored for later use, written directives for posthumous use may be given effect, and subsequently born children may be recognized as legal offspring of the deceased. Post-mortem reproduction with stored gametes or tissue should be honored when the deceased has given specific consent; programs storing gametes, embryos or gonadic tissue from cancer patients should be informed of the options for making advance directives for future use. Whether posthumously conceived or implanted offspring will inherit property from the deceased or will qualify for government benefits will depend on the law of the jurisdiction in which death occurs [29].
3. Concerns about the welfare of offspring resulting from an expected shortened life span of the parent. This concern, however, should not be a sufficient reason to deny cancer survivors assistance in reproducing. Although the effect of the early loss of a parent on a child is regrettable, many children experience stress and sorrow from other circumstances of their birth. The risk that a cancer survivor will die sooner than other parents does not impose an appreciably different burden than the other causes of suffering and unhappiness that persons face in their lives. Protecting such children by preventing their birth altogether is not a reasonable ground for denying cancer survivors the chance to reproduce [29, 30].
4. Concerns about the welfare of children born using gametes frozen after chemotherapy already started.
5. Reseeding of cancer after transplanting cryopreserved tissue.

Future successful production of germ cells *de novo* could have applications in fertility preservation. Sterile gonads would no longer limit reproduction as it will be possible to produce artificial gametes by dedifferentiation of somatic cells.

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Chapter 42

# Legal aspects of fertility preservation

Nanette R. Elster

## Introduction

Give me children or else I die.
*(Genesis 30:1)*

This quote is even more poignant when discussing the reproductive planning and options of a cancer patient or cancer pre-vivor. For purposes of this chapter the term "pre-vivor" refers to one who does not yet have cancer but has an increased risk or predisposition to having cancer, such as an individual who is identified as having a BRCA1 or BRCA2 mutation. The imperative to have children is something that can be both psychological and biological, and it may be further exacerbated by one's own confrontation of mortality. Recent scientific advances have made the once remote possibility of conception following cancer treatment more tangible; however, these advances are not without risks including legal and ethical risks.

Learning that one or one's child has cancer or that one is at a greatly increased risk of developing cancer is life altering. Parents, patients and/or caregivers are bombarded with innumerable treatment decisions, many of which need to be made urgently with little time for deliberation. As such, meaningful discussion about fertility preservation often does not occur. In fact a study published in the *Clinical Journal of Oncology Nursing* of nurses' attitudes regarding fertility preservation revealed that "patients may be strongly focused on survival and their cure and may not want to think about other issues…" [1]. According to a survey by Carlson, the vast majority of surveyed physicians discuss the potential for infertility with patients receiving cancer treatment; however, fewer than 25% responded that they made a referral to a reproductive specialist or provided educational information [2]. Even more striking in the survey was that only 38% of respondents knew that the American Society of Clinical Oncology (ASCO) has developed guidelines on fertility preservation [2]. Another study, published in the *Journal of Clinical Oncology* found that fewer than 25% of men bank sperm and the most common reason for not doing so was that the option was not made known to them [3]. Nearly one half of the men in the study had no recollection of any discussion about infertility before beginning their cancer treatment [3]. What is not clear is whether their lack of recollection is the result of not being provided with the information or the result of the stress and strain of having to cope with a cancer diagnosis and make immediate decisions directly impacting their cancer treatment. This research does suggest, however, that more education of and communication with patients about potential infertility resulting from treatment as well as fertility preservation options is necessary.

Advances in reproductive technology and oncology have made what was once impossible, possible, by allowing young cancer patients extended periods of survival and thus enabling them to contemplate a future with children. Cryopreservation of sperm has been an available option for men since the mid twentieth century [4], but cryopreservation of oocytes has only become an option for women in the last decade or so and is still considered largely experimental despite the proliferation of publicity regarding it. According to the American Society for Reproductive Medicine (ASRM), "[a]lthough currently investigational,…oocyte cryopreservation holds promise for future female fertility preservation, …" [5]. Unlike collection of sperm for cryopreservation, collection of oocytes carries more risk and requires more time potentially delaying cancer treatment.

Numerous other treatments or experimental procedures are also available. According to the ASCO,

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current options for fertility preservation in males include sperm cryopreservation, gonadal shielding during radiation, testicular tissue cryopreservation and testicular suppression; and for females, embryo cryopreservation, oocyte cryopreservation, ovarian cryopreservation and ovarian transposition are some of the available means of fertility preservation [6]. The availability of any given option, however, may be limited by a number of factors including age of the patient, risk of the procedure, prognosis of the patient as well as accessibility to and cost of the procedure. Each of these potential limitations has legal implications as well.

The majority of legal issues related to fertility preservation revolve around decision making of the patient or the patient's guardian: informed consent for both adult and minor patients; participation in a research protocol or undergoing a more standard medical procedure; and future use and disposition of preserved gametes and/or reproductive tissue, including the potential necessity for female patients to utilize the services of a gestational surrogate. These issues are all related to the standard of care for the healthcare professional and thus raise questions of potential liability. Another significant legal issue that impacts patients, regardless of age or gender, is the question of insurance coverage. In this chapter each of these issues will be discussed for both adult and minor patients.

## Informed consent

Informed consent is the embodiment of respect for persons and reflects both a bioethical and a legal construct, applying to both treatment and research. The essential elements of informed consent are the same in both the treatment and research context, and include a discussion with one's physician or investigator regarding the risks, benefits and alternatives to a particular treatment or protocol. Informed consent is not merely a signed document but rather an ongoing dialogue, the purpose of which recognizes "that adults are entitled to accept or reject healthcare interventions on the basis of their own personal values and in furtherance of their own personal goals" [7].

For those unable or incapable of this level of self-determination, such as minors, parents, acting in the child's best interest, will be called upon to make such determinations. The sections that follow will discuss the issues of informed consent in both research and treatment for both adults and minors undergoing cancer treatment in which their future fertility might be compromised.

### ASCO guidelines

In 2006, ASCO recommended that:

> As part of education and informed consent before cancer therapy, oncologists should address the possibility of infertility with patients treated during their reproductive years and be prepared to discuss possible fertility preservation options or refer appropriate and interested patients to reproductive specialists [6].

Unless this is part of the discussion between the physician and the patient or patient representative, it cannot and should not be assumed that the patient is aware of any potential impairment of his or her fertility, let alone any measures that might be taken to preserve his or her fertility if warranted by the patient's prognosis. Once made aware of this possibility, however, the patient should then be provided with information about what, if any, means might be available for preserving fertility and how any such interventions may or may not impact his or her cancer treatment. The ASCO suggests that it is the role of the oncologist to "[a]nswer basic questions about whether fertility preservation options decrease the chance of successful cancer treatment, increase the risk of maternal or perinatal complications, or compromise the health of offspring" [6]. Referrals to reproductive medicine specialists and/or a mental health provider may also be necessary or advisable [6, 8]. Despite this recommendation, however, in one recent study most oncologists in academic centers discuss the risk of infertility with female patients, but rarely make a referral to a reproductive endocrinologist [9].

### Treatment – adults

The legal principle of informed consent was solidified nearly 40 years ago by the US Court of Appeals for the District of Columbia in the seminal informed consent case *Canterbury v. Spence*, which delineated what ought to be disclosed to patients. In that case the court held that:

> True consent to what happens to one's self is the informed exercise of a choice, and that entails an opportunity to evaluate knowledgeably the options available and the risks attendant upon each. The average patient has little or no understanding of the medical arts, and ordinarily has only his physician to whom he can look for enlightenment with which to reach an intelligent decision. From these almost axiomatic considerations springs the need, and in turn the

requirement, of a reasonable divulgence by physician to patient to make such a decision possible [citations omitted] [10].

The ASCO and ASRM recommendations regarding elements to include in the informed consent process are in keeping with the principles set forth in *Canterbury* as that set forth the type of information that one would likely need in order to make an informed choice. Informing patients about potential risks to fertility of cancer treatment, followed by a discussion of fertility preservation options is the starting point. Patients need to also have information about how those options impact their treatment, the potential success of these options when the patient is seeking to reproduce, the cost of the procedures, whether the procedure is experimental and the risks such options may have to their own health as well as that of future offspring [6], [8].

While little has currently been written about the information that should be provided to cancer previvors regarding fertility preservation, many of these same issues merit consideration. For some pre-vivors, the option of fertility preservation is what will enable them to undertake prophylactic measures to reduce their heightened risk of cancer. For example, a young woman with a BRCA1 or BRCA2 mutation who has not yet had children, may be reluctant to remove her ovaries, a prophylactic surgery which would significantly reduce her risk of ovarian cancer [11]. However, if she becomes aware of fertility preservation options, both in clinical practice and research, she may be more comfortable considering this option. As such, not only is it important for oncologists and reproductive endocrinologists to consider when, what and with whom to discuss fertility preservation options, it may also be important for geneticists and genetic counselors to be aware of fertility preservation options as well and to consider disclosing information about these options to those patients who might benefit.

Given the depth and breadth of information that needs to be communicated, reliance on a multidisciplinary team may be the most efficient and expeditious means of communicating the information. Jeruss and Woodruff have recommended that institutions create a multidisciplinary board to aid in "indentifying and communicating options for the preservation of fertility in patients with cancer" [12]. Others also recommend involving clergy and theologians in any fertility preservation program [13]. These are sound recommendations for patients and practitioners given the rapidly changing technology, the paucity of specific legal guidance and the likely increase in patient demand as more individuals become aware of these options.

## Treatment – children

As mentioned above, parents are responsible for making medical decisions for their children. "The general rule seems to be that, unless there exists an emergency, which prevents any delay, or other exceptional circumstances, a surgeon who performs an operation upon a minor without the consent of his parents or guardian is guilty of a trespass and battery" [14]. This decision-making authority is part and parcel of the constitutionally recognized right of parents to the care, control and custody of their children [15]. An historic rationale for giving parents this authority was based on the notion that if anything went wrong with the medical procedure the parents would be responsible for raising the child and thus should make the decision with this awareness [14, 16]. Still another rationale for bestowing the obligation upon parents is that parents are in the best position to protect their child's welfare and to consider what would be in the best interests of the child [17].

Under some circumstances, however, minors can make their own healthcare decisions, particularly around issues of reproduction. States have exceptions for emancipated minors, mature minors and for specific healthcare services [16]. For example, the US Supreme Court in a series of cases found that minors have a right to determine whether to bear a child and the decision to do so should not be vetoed by another; however, some limitation is considered permissible [18, 19]. In addition, legislation exists which allows minors to make treatment decisions regarding sexually transmitted infections without parental consent or notification [20]; obtain information about contraception [21]; and to obtain medical treatment for alleged sexual assault without parental consent [22]. These statutes are in keeping with the general understanding that the right to reproduce is a fundamental one. Further supporting this is the recognition by most states that parents cannot make the decision to sterilize a minor without independent court review [23]. "Sterilization touches upon the individual's right of privacy and the fundamental right to procreate" [24]. While parental failure to consent to the pursuit of fertility preservation likely is not the same deliberative process as consenting to the sterilization of one's child, the consequence for the child might be the same and thus

indicates the need for thoughtful consideration of the child's future reproductive needs and wishes.

Even outside the context of reproductive health, the American Academy of Pediatrics asserts that healthcare decisions involving adolescents and older children should, to the greatest extent possible, involve the assent of the patient [17]. Assent is, in essence, a child's willingness to accept the treatment after having had the treatment/procedures described to him or her in understandable terms appropriate for his or her level of development [17]. A child's unwillingness to assent should be given great weight [17] and in the context of research may, under some circumstances, be binding [25].

In the context of cancer treatment, parental decision making has become increasingly complex when parents are confronted with the option of fertility preservation. In a Technical Report issued by the American Academy of Pediatrics in 2008, the authors acknowledged that for parents "the act of preserving a child's life must take precedence over the preservation of the possibility of that child's ability to have children, although the goals of each are intertwined" [26]. Legal and bioethics expert John Robertson supports parents' decision to preserve their child's fertility "as long as the minor assents and the intervention does not pose an undue risk" [27]. Dolin *et al*. reach a similar conclusion, but consider the question from the perspective of the child [23]. They assert that "[t]o the extent that the minor in question can rationally consider her options and express her preferences accordingly, that should be the end of the matter" [23]. In the event of a conflict between the wishes of parent and child, if the minor is above the age of 14 years and deemed mature enough to give or refuse consent, there seems to be support to allow the minor to do so [3].

While many of the fertility preservation procedures available to adults are also available for children, not all are. This is, in part, due to psychosocial concerns. For example, "Adolescent girls have not been considered to be candidates for assisted reproductive technology, largely because of the psychosocial issues surrounding a delay in treatment and acquisition of donor sperm" [12]. Additionally, parents may, because of religious or cultural beliefs, be uncomfortable with some procedures such as cryopreservation of sperm collected through masturbation [26].

Because parents cannot always remove their own emotions and their own needs to contemplate what the child might desire when he or she becomes an adult, it may become necessary to consider the appointment of a special advocate for the child or have an ethics committee or consultant review the decision. The law tends to be reactive, not proactive, and thus little if any legal guidance exists to dictate what should or should not be done to preserve the fertility of minors. Given the uncertainty, however, thoroughly discussing the issue with the child, if mature enough to understand, and with the child's decision makers is imperative, especially in light of the fact that while "[i]mpaired future fertility is difficult for children to understand [it is] potentially traumatic to them as adults" [6].

How, what, why and when the discussion about fertility preservation should occur with minors and their parents will be somewhat case specific with consideration of a number of factors including but not limited to the age of the child; maturity of the child; family dynamic, particularly the nature of the relationship between the parent and the child; cultural and religious beliefs of the family; child's prognosis; cost; and availability and accessibility of any given fertility preservation option in the patient's geographical area.

## Research – adults

Several fertility preservation techniques are still in the experimental phase, and thus the elements of informed consent for research should be adhered to regardless of the funding source for such research. The goals of research and practice differ and this must be reflected in the consent process. The goal of treatment is to benefit an individual patient whereas the goal of research is to contribute to generalizable knowledge [28]. Based on this distinction, more protections are required of human participants in research. One such protection includes the Code of Federal Regulations' requirement that research involving human subjects be reviewed by an Institutional Review Board (IRB) [29].

The role of the IRB is to determine that the risks to patients are minimized; that the benefits of those participating in the research will outweigh the risks; that selection of participants is equitable; and that informed consent is sought from the participant or his or her representative [30]. The IRB review will include a review of consent documents as well as the consent process to determine whether all of the consent elements required by the Regulations are satisfied. Section 46.116 sets forth the general requirements for informed consent. These requirements include, among other things, an explanation of the purpose of

the research and a description of the procedures; a description of the risks, benefits and alternatives; and a statement that participation is voluntary and that the participant is free to withdraw at any time without fear of compromising his or her medical care [31].

Recognizing the experimental nature of oocyte cryopreservation, for example, the ASRM has issued a Practice Committee Opinion on the essential elements of the informed consent process [32]. Among the elements to be discussed is a description of the procedure or procedures to be followed; the costs, including annual storage fees for the oocytes; the likelihood of success; and the need to determine the disposition of any cryopreserved oocytes in the event of death [32]. These requirements adhere to the requirements set forth in the Code of Federal Regulations. Another important factor is that patients recognize that the goal of research differs from that of treatment and in fact, the goal of research is really not treatment [28].

## Research – children

When it comes to research, children are considered to be a vulnerable population, in no small part due to the long and disturbing history of research abuses involving children [16]. Because of the vulnerability of this population, heightened protection of children participating in research is required by the Code of Federal Regulations. The Federal Regulations distinguish between beneficial and non-beneficial research when the participants are children, in that if the research holds no direct benefit to the minor, then even a "mature minor" as defined by a state law could not consent to the research [16]. With regard to fertility preservation, however, this would not seem to be the case since arguably, the purpose of undertaking any experimental fertility preservation procedure would be to directly benefit the child at some point in the future. This does not, however, mean that such procedures should not be considered experimental.

As with treatment of children, research involving child participants should seek the assent of the minor and "if the child dissents from participating in research, even if his or her parents or guardian have granted permission, the child's decision prevails" [33]. With regard to research presenting minimal risk to the child [34], or research in which there may be more than minimal risk but with a direct benefit to the child, the Federal Regulations require the consent of one parent and the assent of the child [35]. According to Glantz, "The less risky and more beneficial the research seems to be, the fewer the requirements" [16]. And this will seemingly be the case with most of the experimental fertility preservation procedures offered to children.

Robertson posits that since the use of some fertility preservation procedures are "intended to benefit the minor subject, they might be done with the minor's assent and the consent of his or her parents if an IRB finds that the potential benefit of preserving fertility outweighs the burdens of retrieving gametes or gonadal tissue" [27]. Currently, ASRM, for example considers oocyte cryopreservation, ovarian tissue cryopreservation and testicular tissue cryopreservation to be experimental [8]. Additionally, Fallat *et al.* found that "[c]urrent fertility-preservation options for female children and adolescents should be considered experimental and are offered only in selected institutions in the setting of a research protocol" [26]. In this way, the heightened protections of the Federal Regulations will be followed.

## Standard of care?

Is this or should this be the standard of care for oncologists and/or reproductive endocrinologists? If so, could failure to adhere to comply with these recommendations result in potential future liability? The standard of care regarding the information to be disclosed for informed consent for medical treatment can be defined as that information which a reasonable person similarly situated would need to know in order to make an informed decision [28]. Failure to provide the necessary information for informed consent might result in a negligence claim. The elements of a negligence cause of action include: duty, breach of that duty, injury and proximate cause [28]. As the information about fertility preservation continues to grow, its success continues to increase, and the leading professional societies continue to recommend disclosure about fertility preservation there may, in fact, become a "duty" to disclose such information in the treatment context. If this becomes the case, then failure to do so may result in liability if damages result and the failure to disclose the information is the proximate cause of that injury.

# Future use/disposition of gametes and/or reproductive tissue

One cannot overlook the circumstances under which gametes and/or reproductive tissue are being collected and stored when working with cancer patients or

pre-vivors; and, as in any circumstance under which reproductive material is collected and stored for future use, an advanced directive regarding disposition of gametes, embryos and/or reproductive tissue in the event of death, divorce or incapacity must be discussed and documented. Outside the fertility preservation context, this has been at issue in several legal cases involving posthumous reproduction and the use and distribution of previously collected gametic material.

One of the first times this issue was tackled was in the mid 1990s when Judith Hart was conceived from her deceased father's cryopreserved sperm 3 months after his death from cancer. No question existed regarding the child's genetic make up, but when Judith's mother sought Social Security Survivor benefits for her daughter, the child was denied those benefits when the Social Security Administration determined that under the law of the state of Louisiana, where Judith was born, she was not considered a child of the deceased [36]. Several years later an apposite result was reached by the Supreme Judicial Court of Massachusetts. In *Woodward v. Commissioner*, the court found that the children conceived from their deceased father's sperm were entitled to Social Security benefits because their genetic relationship to the decedent was clear and the decedent's affirmative consent to the posthumous use of his sperm was also clearly articulated [37].

Surviving spouses or partners of the deceased as well as parents wanting to utilize a deceased child's gametes to create a grandchild – a legacy of their beloved child – are not far-fetched scenarios and have, in fact, been a reality. In 1997, for example, the parents of Julie Garber, a deceased 28-year-old woman who had cryopreserved embryos comprised of her eggs and donor sperm prior to undergoing treatment for leukemia, sought to enlist the services of a gestational surrogate to gestate and deliver Julie's child [38]. Julie's parents, aged 62 and 68, inherited the embryos upon their daughter's death and then gifted them to Julie's brother and his wife who planned to raise the child [38]. The surrogate ultimately miscarried [39], ending the immediate need to address the legal and ethical dilemmas raised, but this highly publicized news story nevertheless brought to the fore many of the potential legal and ethical quandaries that may arise in the event that disposition determinations are not made at the outset of any fertility preservation treatments. These issues are especially pertinent with the expansion of fertility preservation options.

Because of the panoply of legal and ethical issues that may arise, the ASCO recommends that, "Potential legal issues, such as ownership of embryos and reproductive tissue in the event of a patient's death, divorce or incapacity, should … be discussed by the reproductive specialist" [6]. Additionally, the ASRM, in its Practice Committee Guidelines on the Essential Elements of Informed Consent for Elective Oocyte Cryopreservation recommend that the informed consent process should include information about "[t]he disposition of any cryopreserved embryos not used … in the event of death" [32]. The dispositional options would include, having the material discarded, utilized for research purposes or used for posthumous reproductive purposes [8]. According to the recommendations of the Ethics Committee of the ASRM, "Spouses or family members with legal rights to dispose of a deceased patient's stored gametes or other material should use them for posthumous reproduction only if the deceased had previously consented to such posthumous use" [8]. From a legal standpoint, having that consent in writing would be the most reliable expression of the patient's wishes and would avoid the need to have friends and family members attempt to recall and re-state what it is that the patient would have wanted.

The guidelines set forth by professional societies provide useful guidance and may, as discussed above, serve as evidence of the standard of care; however, they do not have the force of law. Case law and statutory law, though, are beginning to evolve. As posthumous reproduction has continued to occur, some states have developed specific legislation to assist in determining the status of children conceived after a "parent's" death in order to facilitate such determinations as inheritance and parentage. Virginia, for example has enacted a statute which reads:

> Any person who dies before in utero implantation of an embryo resulting from the union of his sperm or her ovum with another gamete, whether or not the other gamete is that of the person's spouse, is not the parent of any resulting child unless (i) implantation occurs before notice of the death can reasonably be communicated to the physician performing the procedure or (ii) the person consents to be a parent in writing executed before the implantation [40].

This language makes it clear that the wishes of the deceased to become a parent posthumously must be expressed in writing, thus stressing the importance of advanced directives when preserving gametes or reproductive tissue.

The complexity of gamete or tissue disposition is further compounded when the patient is a minor and parents or guardians have medical decision-making authority for the child and, presumably, over gametic material or reproductive tissue if the patient dies while still a minor. Parents are likely to inherit sperm, eggs, embryos or reproductive tissue of the deceased minor and thus would have control over the disposition of the material. Given the range of complex scenarios that might arise in this instance, Rosoff and Kastur have recommended "that children and their parents should sign a consent form declaring that they will destroy the materials if the child dies before reaching his or her majority…" [13], as well as having parents and children sign directives regarding gamete disposition in various scenarios. This is very much the same as advanced directives that any patient is required to sign prior to undergoing assisted reproduction in which egg, sperm or embryos might be cryopreserved. Additionally, Fallat *et al*. have recommended that "instructions concerning disposition of stored gametes, embryos, or gonadal tissue in the event of the patient's death, unavailability, or other contingency should be legally outlined and understood by all parties, including the patient if possible" [26]. The enforceability of such a written document by a child is unclear, but, as discussed previously, mature minors do have decision-making capacity with regard to their own reproduction and, thus, should be able to control the disposition of their own gametes.

In any posthumous reproduction scenario, legal, ethical and psychosocial questions abound regarding the dispositional control over gametes or tissue remaining after the death of the gamete or tissue source. Such questions include: legal parentage of a resulting child; inheritance rights of the resultant child, including the ability to receive Social Security Survivor benefits; the psychological ramifications of being conceived after the death of a "parent," especially if that "parent" was a child him or herself at the time of death; and the question of honoring the wishes of the deceased, especially if such wishes have not been clearly expressed in writing.

## Insurance coverage for fertility preservation

Another important issue to discuss with patients and their family members regarding fertility preservation is the cost related to such treatments and the fact that insurance is unlikely to cover not only the treatment but the cryopreservation of gametes as well [27]. Discussion of cost should be included in the informed consent process as this information may be determinative for some patients contemplating fertility preservation.

Currently, at least 14 US states have some type of insurance mandate related to coverage of infertility services [41]. These mandates, however, also impose many restrictions on use, which essentially render them inapplicable to those individuals utilizing assisted reproductive services in order to preserve fertility rather than to currently treat existing infertility. In fact, none of the existing statutes specifically address coverage of fertility preservation and the terms of coverage typically are only applicable to an individual with diagnosed infertility and do not cover cryopreservation. Infertility is most commonly defined as the "inability to conceive after 1 year of unprotected sexual intercourse or the inability to sustain a successful pregnancy" [42]. This definition would exclude many undergoing reproductive procedures to preserve fertility, particularly minors as they have not yet even attempted to conceive.

Yet another potential exclusion presented by the current language of state insurance mandates is a requirement that the insured be legally married, which again would exclude coverage of fertility preservation services for minors and those who are single at the time of undergoing the procedure. Utilization of experimental procedures would pose still another barrier to insurance coverage as no mandate exists to cover experimental procedures.

The issue of cost and insurance coverage for fertility preservation, while seemingly outside the traditional realm of informed consent is highly relevant to patients and parents determination of whether fertility preservation is appropriate for them. With all of these barriers to obtaining insurance coverage, the cost of this prophylactic treatment may be well out of reach for many patients and their families. For this reason it is important to discuss with patients other options for family building following successful cancer treatment, including adoption, egg donation, sperm donation, embryo donation and gestational surrogacy.

## Conclusion

As cancer patients and cancer pre-vivors continue to have better prognosis for long-term survival and

fertility preservation techniques continue to develop the law defining the rights and obligations of patients and providers will evolve. With the goal of improved cancer treatment and identification of genetic predisposition to certain cancers being increased, taking a holistic approach to treating the patient is essential. Fallat *et al*. suggest that "offering the technique might provide some degree of comfort in light of a life-threatening diagnosis, because if offer an optimistic perspective for the future that may conform to a patient-centered philosophy of care" [26]. Such an approach would take into consideration not only the patient's immediate need for treatment and counseling, but also how such treatment and the resultant increased survival will impact his or her life plans which may very well include family building.

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Chapter 43

# Christian ethics in fertility preservation

Brent Waters

The purpose of this chapter is to provide an interpretive framework for examining how Christian theological tenets and convictions might inform an ethical assessment of fertility preservation. In undertaking this task I make no attempt to survey various religions, other than to note that their respective beliefs inspire disparate moral assessments of the medical procedures deployed in preserving fertility. Subsequent inquiry in comparative religious ethics and interfaith dialogue are certainly required given the diverse character of contemporary society, but will need to be pursued by scholars with greater expertise. I also do not attempt to make a systematic or normative assessment of fertility preservation in general, nor do I offer any detailed evaluation of the discrete ethical issues accompanying the particular medical procedure described in the previous chapters. Again, these are important tasks, but beyond the limited scope of this chapter.

My intent, to invoke a crude analogy, is to visit some important theological landmarks along the moral turf in which fertility preservation is embedded. My description of these landmarks is both informed and limited by my perspective as a Christian theologian, but I use terminology which I hope will prove informative to broader religious, secular and professional audiences. In most respects we will be revisiting familiar landmarks, for many of the ethical issues associated with fertility preservation are similar or identical to earlier and ongoing disputes over the ethics of assisted reproductive technology (ART) generally (e.g. [1–3]). The principal contextual difference in this instance is that fertility is being preserved, and thereby assisted, in response to largely non-controversial therapies rather than treating infertility. Given the wide variety of Christian churches and their respective teachings and theological convictions, no universal ethical position on the ethics of fertility preservation in general or the associated medical treatments in particular can be formulated. Consequently, in conducting this inquiry I examine four pertinent theological and biblical precepts; describe four general moral stances along a spectrum of options that may be derived from these precepts; and identify a principal strength and weakness, respectively, of each stance.

## Theological and biblical precepts

The list of precepts examined below is not exhaustive, but they have been selected as examples for how ethical arguments based upon religious beliefs and convictions might be formulated. The first general precept may be characterized as the *procreative mandate*. As reported in Genesis 1.28, God commands humans to be "fruitful and multiply." Theologians have drawn upon this passage in arguing that humans are in general commanded by God to procreate, and that offspring is the premier good of marriage (e.g. [4, 5]). Fecundity is thereby held in high regard as a necessary means of fulfilling this religious obligation. It is in light of this procreative mandate that infertility is often portrayed in the Bible, especially in the Hebrew Scriptures or Old Testament, as a particularly severe curse or tragic circumstance [6].

Although there is a long history of valuing fertility within the Christian tradition, there is no contemporary consensus regarding the ethics of ART in general and fertility preservation in particular. On the one hand, it can be argued that, despite the high esteem afforded to fertility, preserving the biological nature (sexual intercourse) of transmitting life and inviolable structure of monogamous marriage precludes any artificial or technological interventions to either treat infertility or preserve fertility. Specifically, artificial insemination by donor (AID) or by

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husband (AIH), in vitro fertilization (IVF), embryo or gamete storage, pre-implantation genetic diagnosis (PGD) and surrogacy would all be prohibited as violating the biological or marital integrity of procreation. Consequently, this predominantly Catholic teaching forbids any recourse to ART, which in turn also prohibits virtually all of the medical treatments associated with fertility preservation [7]. On the other hand, it can be countered that the so-called biological and marital integrity of procreation is not sacrosanct. The natural and legitimate desire for offspring should not be frustrated by a biological essentialism and highly restricted view of marriage which effectively constrains the freedom to reproduce. This largely liberal Protestant stance contends that using ART to treat infertility is thereby morally permissible in treating infertility and, by extension, so too fertility preservation.

Closely related to the procreative mandate is the second general precept of *stewardship*. Following God's command to be fruitful and multiply, humans are enjoined to exercise a stewardship of the earth's resources (see Genesis 1.28–30). Asserting such dominion or rule, however, is limited for ultimately the earth belongs to God; humans are the caretakers and not the owners of creation. Consequently, such human governance should accord with divinely inspired concepts of what constitutes the larger or general good of creation.

Subsequent theological reflection on the precept of stewardship has developed, among other things, the idea of the common good. Since material goods are finite, the pursuit of the common good may require that the desires of some individuals remain unfulfilled in order to promote a just distribution of scarce goods and services. For example, the desire to be rich is not necessarily wrong, but the desire should remain unfulfilled if its fulfillment results in impoverishing other individuals which in turn diminishes the common good.[1] Likewise, assisting reproduction and preserving fertility might very well be good desires, but whether or not they should be fulfilled needs to be determined within a larger set of social, economic and political considerations. Consequently, the ethics of fertility preservation should not be evaluated solely in terms of personal preferences and therapeutic safety and efficacy, but also in respect to healthcare priorities, costs and accessibility. Given the general healthcare needs of civil community, does preserving the fertility of relatively few individuals justify the allocation of scarce medical funding and personnel in achieving this goal?

The third precept is derived from the doctrine of the *incarnation*. The Gospel according to St John asserts that the Word became flesh, a reference to Jesus Christ (see John 1.1–18). The central tenet of this doctrinal teaching is that in Christ, as the second person of the Trinity, God became a human being. This act in turn affirms the embodied nature of human beings and consequently the finitude of the human condition. Bodily health is therefore not a matter of indifference. Subsequent theological reflection has affirmed the goodness of the body despite frequent heretical attempts to disregard or malign the body as a mere vessel, or worse, a prison of the soul. In Beth Felker Jones' evocative words, "In the Christian tradition, the temptation to denigrate the body has been continually reasserted and consistently rejected"[8].

The doctrine of the incarnation inspires a Christian affirmation of the human body, but no obvious ethical stance is forthcoming regarding modern health care in general, or assisted reproduction and fertility preservation in particular. Although the life of embodied human beings is highly valued and respected, efforts to preserve or reproduce life are relative rather than absolute [9]. For example, there is no corresponding moral duty to use every means possible to extend the life of dying individuals for as long as possible should such efforts prove unduly onerous or futile, though euthanasia and assisted suicide are generally proscribed [10, 11]. Similarly, the biological means of reproducing human life is held in high esteem, but it is again a relative rather than absolute good to be pursued. An infertile couple, for instance, incurs no moral obligation to employ every available medical treatment. As noted above, a Catholic couple would be prohibited from utilizing ART and could fulfill their natural parental desires through such alternatives as foster care or adoption [12, 13]. Many Protestant couples, however, believe they are free to utilize or refrain from utilizing ART in treating their infertility, which would presumably extend to attempting to preserve or not preserve fertility as well. This expansive range of options is derived from the theological teaching of the incarnation which while affirming embodiment, and derivatively reproduction, also indicates that with the birth of Jesus the urgency

[1] I am not convinced that this zero-sum argument regarding the creation of wealth is necessarily correct.

of the procreative mandate has been effectively diminished [14].

The fourth precept entails *healing* and *love of neighbor*. The Gospels report that Jesus performed many miraculous healings. Given the doctrine of the incarnation these acts are not surprising, for in affirming the embodied nature of human life ameliorating the pain and suffering that is inherent to the life of finite and mortal creatures is also an act of love and compassion. Moreover, Jesus commands his followers to love and care for their neighbors in need (see Matthew 22.34–40), especially those suffering from illness, disability or injury (see, e.g., Luke 10.25–37). In addition, Jesus is portrayed as keeping company with the sick and infirmed, especially those whose conditions have made them outcasts from the larger community.

The complementary images of Jesus as both healer and suffering servant have informed the subsequent theological and moral tradition, which affirms the importance of medicine and health care [15]. Christians have been instrumental in establishing hospitals and were early proponents of medical research. This religious commitment to relieving the human condition is exhibited not only in treating those suffering illness and injury [16], but is extended, by many theologians and official church teachings, to include treating infertility and prenatal screening and monitoring in order to prevent suffering [17, 18]. Presumably this expansive support of ART could easily embrace fertility preservation. Moreover, modern medicine exhibits in a highly visible manner the love for neighbor in the contemporary world, embodying Jesus' roles as healer and suffering servant. In treating or preventing disease and injury the suffering of the neighbor is alleviated, and, more importantly, medicine represents a moral commitment that the ill and infirmed will not be abandoned by the civil community [19]. Arguably a case can be made that preserving fertility is consonant with the precept of healing and love of neighbor.

The preceding brief summaries of the theological and biblical precepts of the procreative mandate, stewardship, incarnation and healing and love neighbor provide some useful starting points for examining how Christians might assess the ethics of fertility preservation. As these summaries suggest, however, these precepts do not lead to a common moral position or stance. Although most Christians would affirm, in varying ways, these precepts, subsequent ethical reflection and discernment can lead to highly disparate assessments of fertility preservation. The next section examines the range of these ethical assessments and how they might be formulated.

## Moral stances

The following four ethical stances are derived from the theological and biblical precepts summarized in the preceding section. Each stance demonstrates how religious beliefs and convictions might inform particular ethical assessments of fertility preservation. These stances do not reflect arguments promulgated by a specific church or theologian. Rather, they serve as heuristic devices that demonstrate varying and often conflicting assessments. Furthermore, these stances disclose how theological and biblical precepts may be interpreted and applied in a variety of imaginative ways in constructing a moral argument. As will be seen, there is no given correspondence between a particular precept and a particular stance regarding the ethics of fertility preservation. It should also be noted that these stances do not exhaust the possible options that could be formulated, but rather serve as examples along a spectrum ranging from prohibition to encouragement.

In each instance I make the following assumptions: An individual is facing the prospect of a therapeutic application that may result in the loss of fertility. The various treatments that could be applied in attempting to preserve fertility are accessible, relatively safe and potentially efficacious and adequate funding is readily available. All legal safeguards and recognized ethical practices and procedures are followed such as informed consent, protection of minors and proper authorization and oversight of experimental protocols. In addition, I make no attempt to assess the adequacy of the various moral stances, especially in regard to their respective interpretations and applications of theological and biblical precepts and coherency of argument, nor do I offer any counter interpretations or arguments. Rather, I allow each stance to stand in its own right in order to identify various points along a spectrum of options concerning the ethics of fertility preservation that are based upon selected religious beliefs and convictions. Furthermore, it should be noted that in some instances these arguments do not accept medical designations of embryonic development, for example, the difference between "pre-embryos" and "embryos," as implying any inherent normative content. It is important

for medical practitioners to keep these potential discrepancies in mind when dealing with some patients who may exhibit some reluctance in pursuing fertility preservation. In short, when doctors and patients refer to an "embryo," they may not, in some instances, be referring to a common perception. Although the religious reservations of these patients need to be honored, they do not necessarily call into the question either the morality of the procedures or humane intentions motivating their development and deployment.

The first stance may be characterized as *preserving the natural reproductive process*. This stance draws heavily upon the precepts of the incarnation and the procreative mandate. The divine affirmation of the embodied nature of human existence necessarily entails the need of human beings to perpetuate themselves from one generation to the next; hence God's command to be fruitful and multiply. This affirmation and command, however, imposes constraints in exercising an accompanying stewardship. In affirming their embodiment and obeying the command to procreate, individuals are not free to do whatever they might want. The affirmation and mandate acknowledges and consents to the frailty and limitations of the human body, otherwise stewardship is distorted into a form of mastery that rightfully belongs only to God. Although this stance does not reject modern medicine in general, indeed it is seen as an important means of excising the stewardship of God's creation and expressing the love of neighbor, it too is limited to restoring the natural health of the body.[2]

Given these religious presuppositions, the morality of fertility preservation is assessed largely, though not exclusively, in respect to whether or not it preserves or violates the natural reproductive process. Consequently, fertility preservation should not be used in many, if not most, instances because the treatments employed violate this natural integrity. Similar to the objections against ART, gamete and embryo storage are illicit because of the means required to procure the gametes and embryos. Although such medical treatments are motivated by the legitimate desire to treat infertility resulting from other therapeutic applications, this motive does not justify bypassing the natural method of procreation. The creation and storage of embryos is particularly objectionable since it might entail the destruction of unneeded and, in the case of PGD, unwanted embryos. The ensuing destruction of embryos fails to exhibit a proper love of weak and vulnerable beings.[3] This stance, however, would presumably have no objections to repositioning ovaries when undergoing radiation treatment in the lower abdominal area. No attempt is being made to bypass the methods of natural reproduction such as extracting oocytes, nor are any embryos artificially created or willfully destroyed. Performing a radical trachelectomy in treating cervical cancer would presumably be illicit since artificial means of initiating pregnancy following treatment would be required. Similar objections could also be raised against the experimental procedure of ovarian tissue cypopreservation if IVF-created embryos are implanted. In short, these treatments, with the exception of ovary repositioning, are illicit not only because they violate the integrity of the natural reproductive process but also because they represent an improper stewardship of medicine as they seek to transcend rather than restore natural health, thereby failing to honor the limitations of embodiment which are affirmed in the incarnation and presumed in fulfilling the procreative mandate.

The principal strength of this first stance is its seriousness concerning the embodied nature of human beings. Since the incarnation affirms embodiment, the inherent finitude and mortality set integral and delimiting conditions that should be honored. To be embodied, therefore, entails a natural reproductive process which may be disabled by disease, dysfunction or therapies treating a condition unrelated to fertility. Although one may sympathize with individuals who are infertile or may lose their fertility, this does not justify recourse to technologies which bypass the natural reproductive process. The principal weakness

[2] The description of the basic religious principles underlying this stance draws upon selected themes explicated in relevant Catholic social teaching. See, e.g., *Donum Vitae* and Paul VI [20]. For related Protestant themes see, e.g., Mitchell *et al.* [21].

[3] Most often this argument is based on the belief that since life begins at conception the resulting embryo is a person, and therefore should be given full moral regard and protection. See, e.g., Grisez [22] and Meilaender [23]. It can also be argued that even if embryos (or fetuses) are not persons their status as human beings should nonetheless be sufficient for protecting them against willful destruction. See, e.g., Grant [24, 25]. In addition, even if agnosticism is invoked regarding the moral status of the human embryo a variety of ethical arguments regarding the protection and ethical treatment of prenatal life can still be made. See, e.g., pertinent chapters in Waters and Cole-Turner [26] and Waters [27].

is its appeal to a biological essentialism that is not applied consistently in respect to other medical practices. Given their status as finite and mortal creatures, humans necessarily suffer the natural effects of disease, dysfunction and degeneration, yet there are presumably few, if any, moral objections to treating these conditions. It is not clear why such rigid prohibitions are set regarding the reproductive process in comparison with other organic systems. The contention that ART, and thereby fertility preservation, attempt to bypass rather than restore natural fertility does not resolve this inconsistency. Using suppressants in conjunction with organ transplants, for instance, bypasses the natural immune system, yet few, if any, theologians would now argue against these procedures in terms of violating the so-called natural integrity of the immune system.[4]

A second stance entails assessing the ethics of fertility preservation in light of larger *social priorities*. The precept of stewardship carries the heaviest weight in formulating this moral perspective and evaluation. Humans do not exercise their stewardship of creation as autonomous individuals, but through cooperative social and political relationships. Technology in general and medicine in particular have undoubtedly assisted humans in fulfilling this responsibility, but their development and deployment should be determined in respect to larger social and political priorities rather than merely satisfying individual desires. The common good and the needs of the many should trump the interests of the few. This moral commitment to the common good affirms the precept of the incarnation by recognizing the finite character of human life within the finite constraints that are imposed by creation. This scarcity must be taken into account in ensuring that a just distribution of goods are services is pursued in a manner which proves most beneficial to the greatest number of people. This same principle holds true in the allocation of scarce medical resources. The relative urgency of the procreative mandate, therefore, should be determined in light of contemporary economic and political concerns related, for instance, to population growth, and how these concerns are addressed in healthcare policies.[5]

Given these religious assumptions it can be argued that although fertility preservation need not be prohibited, the practice should nonetheless be discouraged.[6] Although there is nothing necessarily objectionable to the various treatments employed in preserving fertility, it marks a costly expenditure of scarce medical resources that serves a relatively small segment of the population.[7] These resources should instead be deployed in addressing basic healthcare needs of the broader population that is often deprived of such care due to costs and limited accessibility, thereby promoting the common good rather than serving the interest of a few individuals. Moreover, such a policy or strategy is more in line with Jesus' role as healer and suffering servant, which were most often performed for the sake of the poor and destitute. In addition, given the array of social, economic and political problems associated with overpopulation, it is hard to justify the allocation of funds, the time of healthcare personnel and technologies to meet the needs of relatively few individuals. The ethical issue at stake is not so much to preserve fertility (or treat infertility), but to ease the longing of childless couples or individuals which can be addressed through such options as foster care and adoption. This approach would have the additional benefit of providing parental care for orphans, abused and neglected children, thereby once again promoting the common good, as well as recognizing the need for tempering the urgency of the procreative mandate given the pressing need to control population growth and caring for needful children. In short, although one may be sympathetic with the desire of individuals to preserve their fertility, a faithful stewardship of creation requires that more pressing healthcare needs are given priority. Consequently, fertility preservation should be discouraged as an unwarranted consumption of scarce medical resources.

[4] Most of the ethical disputes now focus on such issues such as fair access to scarce organs and public policies regulating donors.

[5] In describing the basic principles of this stance I have borrowed, rather loosely, from some prominent themes drawn from what may be described as Christian environmentalism. See, e.g., Fern [28] and Scott [29]. In respect to setting social priorities governing the development and distribution of medical resources, see, e.g., Cahill [30].

[6] This discouragement could be accomplished, e.g., through a combination of informal social and religious disapproval, refusal by public and private insurance carriers to cover costs and other economic disincentives.

[7] The same objection could also be raised against ART in general. An individual holding this stance could also be dedicated to preserving the integrity of the natural reproductive process as described in the previous moral stance.

The principal strength of this second stance is the recognition that medicine and health care cannot be separated from larger economic and political considerations. A medical decision is never made in isolation from broader social contexts, as demonstrated by determining which treatments and procedures are and are not funded by public and private insurance carriers. Given limited economic resources, as well as other ethical concerns such as overpopulation, good stewardship requires limiting the provision of medical treatments that affect a relatively small segment of the population. Again, one may sympathize with individuals desiring to preserve their fertility, but it should not be granted much priority, or even discouraged, given more pressing and expansive healthcare needs. The principal weakness is its implicit paternalism. Invoking a greater common good often entails an appeal to abstract moral principles that are divorced from actual practice. This disjuncture in turn effectively masks the imposition of the emotive values and preferences of some over those of others; those in a relative position of power know what is best for everyone, thereby corrupting stewardship into an exercise of behavioral control [31]. Yet it is not clear why fertility preservation should be discouraged on the basis of utilizing scarce medical resources contributing to overpopulation when such a small segment of potential patients are at stake. Rather, discouraging fertility preservation by appealing to larger social priorities may effectively serve as a more troubling wedge argument. If the common good is promoted by limiting births in general and the births of individuals with potentially chronic conditions in particular since both place strains upon limited healthcare resources, then discouraging fertility must been seen within the context of social and political agendas of utilizing medical technologies to control both the quantitative and qualitative outcomes of reproduction (e.g. [32, 33]).

A possible third moral stance can be characterized as one of *freedom of choice*. The embodiment affirmed in the precept of the incarnation does not diminish the need for moral agency and the personal responsibility it entails. Constricting or denigrating the freedom to choose among various possible options effectively denies the human dignity which the incarnation affirms. Stewardship, therefore, should be directed toward enabling the concrete and varying goods of individuals rather than promoting an abstract common and collective good. In respect to medicine and health care, it should be noted that Jesus was not required to either heal or keep company with the ill and infirmed, but freely embraced his roles as healer and suffering servant. Likewise the Good Samaritan was not compelled to stop and render aid but chose to do so. Similarly, although the urgency of the procreative mandate may be muted in light of contemporary population concerns, it has not been rescinded and individuals should be free to make responsible reproductive choices. To portray individual freedom and personal responsibility as antithetical to the common good, particularly in respect to health care and procreation, is to effectively eviscerate the very meaning of ethics, especially Christian ethics, since the love of neighbor expresses moral action based upon liberty as opposed to compulsion.[8]

Given these basic religious convictions, this stance would permit fertility preservation while neither encouraging nor discouraging its use. Since the decision to either employ or forgo fertility preservation is a matter of personal discernment and a wide variability of choices are to be expected. On the one hand, for instance, if an individual does not believe that the various medical treatments and social circumstances are morally objectionable then presumably there would be no compelling ethical reason to refrain from attempting to preserve one's fertility.[9] So long as it is determined that, for ART and the potential destruction of unneeded embryos, these procedures are not jeopardizing basic healthcare provision or substantially contributing to problems associated with overpopulation, then an individual is free to pursue fertility preservation since it does not violate the theological and biblical precepts outlined above. On the other hand, if an individual believes that the medical treatments and social conditions are morally troubling then presumably there are strong ethical reasons to refrain from attempting to preserve one's fertility. If it is discerned that ART, the potential destruction of unneeded embryos or that the provision of these treatments are unjust given the lack of basic healthcare provision or overpopulation concerns, then an individual would choose not to pursue fertility preservation since

[8] Although Victor Claar, Robin Klay and Michael Novak do not address healthcare issues directly, their respective accounts of economics help to explicate this broadly "free market" account of stewardship. See Claar and Klay [34] and Novak [35].

[9] An individual would still be free not to pursue fertility preservation as a matter of personal preference.

doing so would violate relevant theological and biblical precepts. It should be noted that, although Christians come to conflicting conclusions regarding the ethics of fertility preservation, the source of moral authority resides in the conscience of the individual believer rather than compliance with external sources. The act of moral discernment and action stems from the will of the individual rather than obedience to prohibitions imposed by the larger community.

The primary strength of this third stance is that it places the weight of moral responsibility upon the individuals most directly affected by the treatments in questions. Consequently, individuals should be free to make whatever choices they might make regarding fertility preservation, so long as they do not violate their own religious and moral convictions or that such decisions do not demonstrably harm others. In the absence of freedom of choice, how else can individuals affirm their embodiment, pursue procreation and exercise their stewardship as the responsible beings they were created to be? The question of whether or not to pursue fertility preservation is best left to patients facing the prospect of undergoing therapies that may compromise their fertility in consultation with appropriate medical expertise. The primary weakness is a diminished understanding of freedom that is reduced to license. Freedom is not merely the absence of external constraints against the will of individuals pursuing their respective reproductive interests. Rather, freedom results from limitations necessarily imposed by various relationships [36]. Responsible choices regarding fertility preservation cannot be made in isolation from the interests of partners, spouses, family members, medical personnel and healthcare institutions, religious communities and the civil community. In the absence of these considerations, freedom is eviscerated into a fictional autonomy that potentially distorts the purported affirmation of embodiment, pursuit of procreation and stewardship into self-indulgence. Moreover, coupling freedom with such a diminished understanding of moral autonomy exacerbates the more troubling aspects of the so-called "procreative liberty" pervading contemporary society by adding fertility preservation to a growing list of reproductive options designed to bypass biological limitations and social inequalities [37].[10]

A final possible stance may be characterized as a *technological affirmation of life*. Although the procreative mandate has lost its urgency in the modern world, using medicine to preserve fertility may nonetheless serve as a witness to the goodness of life in a dominate "culture of death" [41].[11] If medical technologies are routinely used to prevent conception, destroy embryos and abort fetuses, why can't they also be used to assist the birth of children thereby promoting a culture of life? In this respect, fertility preservation serves as a powerful countervailing witness to the dominant culture. Such a witness upholds the incarnation's affirmation of embodiment, while also enabling a stewardship of medical resources oriented toward securing the good gift of life. Moreover, using medicine to preserve fertility is consonant with Jesus' ministry of healing and ameliorating suffering.

Based on this admittedly highly speculative interpretation of the theological and biblical precepts discussed previously, this stance would not only permit but would encourage fertility preservation; there is not only permission but an implicit imperative to preserve fertility.[12] There are, however, some moral constraints that should be honored in pursuing fertility preservation. Presumably the techniques of repositioning ovaries, performing a radical trachelectomy, ovarian tissue cryopreservation, artificially extracting and fertilizing gametes, storing and implanting embryos and surrogacy are permissible. Yet if these treatments are being employed, in part, as a witness to enabling a culture of life, then provisions need to be in place for ensuring that unneeded embryos are not destroyed, a goal that could be accomplished through embryo donation or adoption (e.g. [44]). In addition, PGD would also be prohibited since it implies the possible destruction of embryos with genetic or chromosomal abnormalities.

[10] For a religious account that both criticizes and utilizes, albeit often obliquely, many of the arguments propounded by Robertson, see Peters [38]. For a critique of Robertson, and more broadly the concept of procreative freedom, see Meilaender [39]. For a critique of both Robertson and Peters, see Waters [40].

[11] It should not be construed that John Paul II or Catholic social teaching and moral theology would endorse the hypothetical stance I am describing.

[12] I am not aware of a Christian theologian or ethicist making this kind of imperative argument. Although Ronald Cole-Turner does not address the question of fertility preservation specifically, he offers some broader themes, particularly in respect to human genetics, regarding the "redemptive" uses of medical technologies. See, e.g., Cole-Turner [42, 43].

The principal strength of this fourth stance is its embrace of human ingenuity accompanying the affirmation of embodiment. Technology is what makes *Homo sapiens* into human and humane beings; to a large extent humans are rightfully becoming *Homo faber* (e.g. [45]). Technology in general is a significant means for exercising stewardship, and medical technologies in particular for pursuing healing and ameliorating suffering. Moreover, fertility preservation in conjunction with ART provides a refreshing and powerful witness to life in a culture of death in which all too often medicine is used to prevent or destroy life. Consequently, provided that the moral constraints of avoiding embryo destruction and PGD are honored, there are not only no compelling reason why fertility preservation should be either discouraged or greeted with indifference, but should instead be encouraged. The principal weakness of this stance is that it assumes that greater technological development is synonymous with moral, social and political progress. It fails to recognize sufficiently the often unintended and unforeseen evil affects accompanying this so-called progress. This is not to simply parrot the simplistic slogan of technological neutrality in which tools and instruments can be used for good or evil purposes; a scalpel, for example, can be used for surgery or to commit a murder. Rather, it fails to acknowledge the extent to which modern technological development itself shapes or misshapes the moral vision (e.g. [46–50]). In respect to medicine, for example, the patient becomes subtly transformed into an artifact of medical techniques, or in respect to ART, and by extension fertility preservation, distorts procreation into reproductive projects. In short, encouraging fertility preservation may in the long run prove to be one more little piece in forming a *Homo faber* that is not necessarily comprised of more humane individuals.

## Concluding remarks

As the preceding section demonstrates, a singular or universal Christian assessment of fertility preservation cannot be formulated. There are, rather, multiple assessments. This wide variety should not be surprising given the historical divisions among Christian churches in respect to doctrine and practice. Nor should it be presumed that these divisions are uniquely manifested in assessing the ethics of fertility preservation. Rather, ethical concerns over fertility preservation represent continuing disputes which were present in the early development of contemporary bioethics, particularly in respect to such issues as informed consent, experimentation, organ transplantation, genetic manipulation and beginning and end of life issues (e.g. [51–55]).[13] Given these early theological contributions to the burgeoning field of bioethics, it is also not surprising that the moral stances described in this chapter may share some similarities with prevalent secular, and often conflicting, philosophical and ideological orientations. The preservation of the natural reproductive process stance, for instance, draws heavily upon deontological moral theory, whereas the stance stressing social priorities is utilitarian and communitarian; the stance emphasizing freedom of choice is libertarian, while the stance encouraging fertility preservation as an affirmation of life expresses a technological progressivism.

It is important in treating patients and formulating policy guidelines to keep this wide ranging and overlapping diversity in mind. Although Christians may hold general biblical and theological precepts in common, varying interpretations will lead to varying ethical assessments of fertility preservation. Moreover, the range of precepts and resulting moral stances described in this chapter has been illustrative rather than exhaustive. There are a number of other pertinent doctrines and resulting stances that could have been explored. Consequently, there is a need for further and more detailed investigation not only within the various Christian traditions, but, more importantly, also in respect to other religious traditions and communities if ethical assessments of fertility preservation are to take into account the full and rich diversity of the contemporary world in which medicine is now practiced.

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