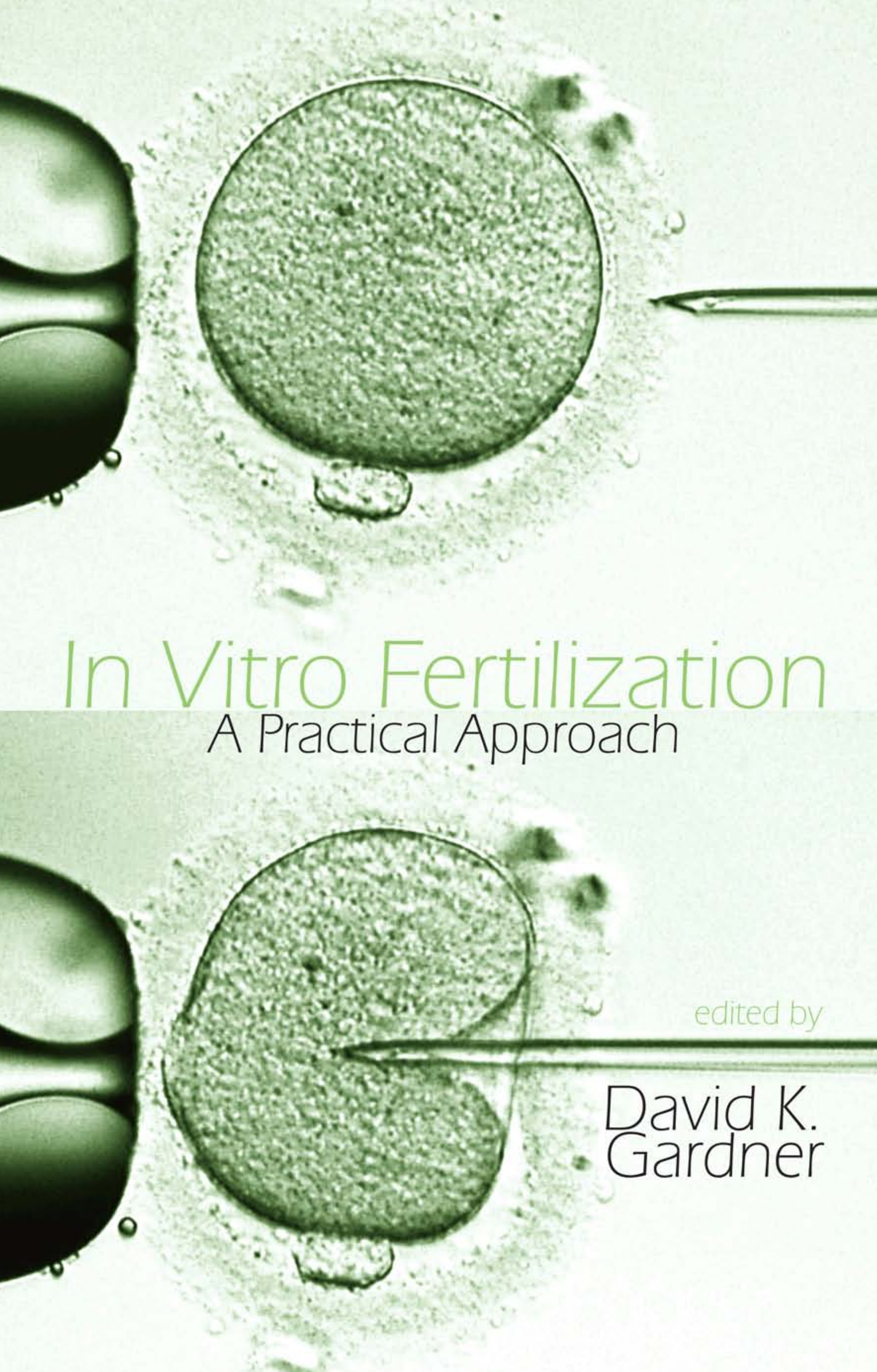
In Vitro Fertilization
A Practical Approach
edited by
David K. Gardner

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A Practical Approach

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To My Family

Preface

The past three decades have witnessed significant advances in the field of assisted human conception. Following the remarkable perseverance and triumph of Robert Edwards, Patrick Steptoe, and Jean Purdy, numerous scientists and physicians from around the world have worked to develop more effective and safer procedures to treat infertile couples. Along with improvements in the areas of ovarian stimulation, embryo culture, and cryobiology, we have seen the introduction of assisted fertilization through intracytoplasmic sperm injection, the development of techniques to remove and perform genetic analysis on polar bodies or blastomeres, and the enhancement of methods for assessing the viability of the developing conceptus.

It is the aim of this book to not only to review the achievements of the biomedical community involved in assisted conception, but also to highlight ongoing and potential future treatments and procedures. Throughout this body of work is the premise that basic science often derives tremendous advances in medicine. This text also offers much practical information with which one can readily translate theory into clinical practice. To this end, the chapters found within have been written by acknowledged pioneers and experts in each respective area.

Together with the advancements in assisted human conception should come careful monitoring of outcomes, including the children conceived.

Outcomes research is well accepted by the reproductive biomedical community and is discussed within this text. While no single volume can adequately cover the enormity of this diverse field of reproductive medicine, this book will be of value to clinicians, cmbryologists, scientists, and all students of biomedical sciences.

David K. Gardner

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1

In Vitro Fertilization: The First Three Decades

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The birth of the world's first baby born as a result of in vitro fertilization (IVF) in July 1978 was by no means a chance event. Indeed, in the long evolution of reproduction, conception by IVF represents the end of a continuum which originated with childbirth wholly dependent on chance but which today is almost exclusively under human control. Today, nearly all forms of infertility can be treated by the various techniques of assisted reproduction, which are now responsible for the birth of around two million children worldwide.

THE HISTORY OF THE PAST

Although the origins of our medical knowledge of human reproduction are usually attributed to Hippocrates, so often described as the "father of medicine," we do know that in the fifth century B.C. it was believed that both males and females each produced two seminal liquors, one stronger than the other; a blend predominantly with the former would produce a male offspring, with the latter a female. In the following century, Aristotle

proposed that the first stage of a human being was indeed the egg found in females. Sperm had the power to give that egg its shape; the male would bring immaterial strength, the female material substance. For centuries, people lived with this concept of pre-formation, even after De Graaf described the follicle in 1672 and, at the same time, Leuwenhoek the spermatozoa. Only in 1875 would Hertwig demonstrate in the sea urchin that only one sperm cell would penetrate the egg to achieve fertilization.

In 1786, Hunter performed the first artificial insemination in humans, and in 1866 Sims the first donor insemination. In 1833, the cytologist Van Beneden demonstrated that gametes had only two chromosomes in the ascaria. The two chromosomes of the male nucleus would join with the two chromosomes of the female to form the nucleus of a new zygote, thereby laying the foundations for the discovery of the hereditary principle. In 1903, a Danish pharmacist, Johannsen, coined the term "gene," from which Bateson 3 years later defined the new science of "genetics." Almost 50 years later, in 1953, Watson, Crick, and Wilkins discovered the double helic structure of DNA and in 1956 Tijo and Levan identified 46 chromosomes in the human.

Equally important were the advances made by gynecologists in their understanding of the physiology of reproduction. By observing the effects of ovariectomy, they were able to explain the function of the ovary and in particular the menstrual cycle; the first treatments were developed as a result of injecting extracts of ovarian tissue. The concept of "hormone" activity was proposed by Baylin in 1904, and the subsequent discovery of the different hormones persisted throughout the rest of the 20th century.

1950–1978

Studies of animal and then human fertilization began in the second half of the 20th century. In 1954, Thibault achieved the first fertilization in vitro in the mammal (in the rabbit); the following year, Chang (1,2) succeeded in growing rabbit embryos derived from oocytes fertilized in vitro, and in 1959 achieved a live birth by transfer of an in-vitro-fertilized oocyte. In 1965, Edwards (3) determined that human oocytes removed from ovarian biopsies required 37 hr to complete their maturation in vitro.

This time was also the beginning of the gynecologist's interest in infertility. It was in 1959 that the first Congress on Infertility was held in New York. Five years earlier, in 1954, the first human pregnancy derived from frozen sperm was achieved, and the following year Pincus (4), who at the time was best known for his (unsuccessful) attempts to fertilize human oocytes in vitro, published the first results on hormonal contraception (for Enovid®, Searle Pharmaceuticals). In 1958 and 1960, Gemzell and Lunenfeld obtained the first pregnancies following treatment with human pituitary gonadotrophin (hPG) and human menopausal gonadotrophin (hMG),

respectively (5,6). In 1961, Klein and Palmer (7) described the first aspiration of a human oocyte during laparoscopy.

However, throughout this time there was also a man working to achieve in humans what had seemed possible from work in animal models, much of it his own work in mice: IVF and embryo transfer. Eventually, in his scientific rigor and disciplined success, this man would change the face of human reproduction, demonstrating throughout persistence, self-denial, and exceptional confidence. This man was Edwards.

Edwards had completed his Ph.D. in 1958 on developmental genetics in mice. His studies, using diakinesis and metaphase-2 as markers, had shown that mice needed around 12 hr to achieve oocyte maturation, but now, as his work progressed from mouse models to the human, it was clear that human eggs required much longer. However, at the same time he and colleagues in Glasgow had produced the world's first embryonic stem cells from rabbit embryos. Intrigued by the therapeutic potential of these stem cells, Edwards turned to the maturation and fertilization of human oocytes in vitro—as a source of stem cells and for other research purposes. And it was from this work with human embryos—during an intense 6-wk period at Johns Hopkins Hospital in Baltimore—that Edwards found that human oocytes required 37 hr to reach full maturity, and thus 35–40 hr after ovulation before insemination could be carried out. By 1969, working with Ph.D. student Barry Bavister, Edwards was able to fertilize human eggs without any obvious need for sperm capacitation.

It was at this time—in 1967—that one of us (Cohen) first met Edwards. Both (Cohen and Edwards) were attending a conference on immunology in reproduction in Bulgaria. We met again in 1972 at an IFFS Congress in Tokyo, and here we talked of the possibilities of IVF in humans. At least, I listened, as he explained his vision of the future of human reproduction—IVF, cryopreservation, preimplantation, and genetic diagnosis. I asked myself if he was serious, but I quickly understood that his was the vision of a true prophet.

Edwards had tried unsuccessfully to collaborate with clinicians in Cambridge and London to supply him with human oocytes. Frustrated in these efforts, he thus turned to the United States and in 1965 had joined Georgeanna and Howard Jones at Johns Hopkins where ovarian tissue (from wedge biopsies) was more readily available. And it was here, during this 6-wk working visit, that he obtained human oocytes, confirmed the precise timing of human oocyte maturation. Back in the United Kingdom, his clinical collaborations continued to prove fruitless, until his chance meeting at a London conference with the gynecologist Steptoe. At the time Steptoe was working in the small northern town of Oldham and already had much experience in the surgical use of laparoscopy. Steptoe immediately agreed to collaborate with Edwards, and so began—in 1968—the partnership that would leave such a lasting legacy in reproductive medicine.

The story of Edwards and Steptoe is well known, but for them it was also a difficult one—the long drives of Edwards from Cambridge to Oldham (180 miles each way), the laparoscopic recovery of oocytes from the ovary, the start of embryo transfers in 1971, ovarian stimulation with hMG, clomiphene, luteal support, and constant failure—until the first ectopic pregnancy in 1975. Finally, despite accusations of malpractice by some U.K. colleagues and after 32 embryo transfers, their first healthy pregnancy was achieved with the birth of Louise Brown on July 25, 1978 (8).

I was surprised that the announcement of the world's first IVF birth was received in such a variety of ways. Certainly, there were very few people in the world who immediately understood the huge importance of its scientific achievement. Many doubted it, or did not even pay it much attention. I remember that I made the trip to London in early 1979 to hear Edwards and Steptoe report their medical and scientific success to the Royal College of Obstetricians and Gynaecologists, and I remember, too, the discussions and doubts when I arrived. However, after their precise and somewhat unsettling lecture (both the biologist and the clinician presenting data), any doubts in the audience evaporated and the meeting ended to the tune of "For he's a jolly good fellow...."

Immediately after the birth of Brown, the attention of Edwards and Steptoe turned to extending their clinical work, but their progress was halted by the retirement of Steptoe from Britain's nationalized health service. It took two more years before an alternative private service could be set up at Bourn Hall near Cambridge, which in time would become one of the most progressive and best known in the world.

However, while Edwards and Steptoe planned their move to Bourn Hall, other groups throughout the world were inspired by the U.K. success and set about their own efforts to repeat it. Like Edwards and Steptoe, they were contemplating a treatment for tubal infertility, with the idea that IVF would circumvent the tubal blockage if tubal surgery had failed.

1978–1982

The embryo that became Brown was derived from a natural—and not stimulated—cycle. Thus, with the success of Edwards and Steptoe showing the way, the predominant scenario of these first IVF attempts was the natural cycle, determination of the luteinizing hormone (LH) peak and follicle puncture during laparoscopy. There was also a new demand for the development of culture media.

In Australia at the time there was already a long history in reproductive medicine. By 1970, Prof. Wood had established a combined research team in Melbourne involving the Royal Women's Hospital and the University of Monash. Johnston was the Medical Director at the former, while Leeton and Talbot comprised the medical staff at the latter, with

Lopata and Trounson handling the biology. This joint group was working with hormonally stimulated IVF cycles throughout the mid-1970s, using hPG or clomiphene and hMG. However, following the birth of Brown, the Melbourne group also turned its attention to the natural cycle. Improvements in culture media were initiated by Trounson, while the development of Teflon-lined catheters by Buttery and Kerin improved the technique of embryo transfer. Australia achieved its first IVF birth—the third in the world—in June 1980 when Candice Reed was born at the Royal Women's Hospital.

In June 1978, Howard and Georgeanna Jones had retired from Johns Hopkins—where Edwards had joined them for his 6-wk working visit in 1965—and had been asked by Andrews to set up a division of reproductive medicine at the Eastern Virginia Medical School in Norfolk. They began their IVF program in 1980, but, following 41 laparoscopies to collect oocytes, they had achieved embryo cleavage in only 13 patients, and no pregnancies following transfer.

In 1981, Georgeanna Jones proposed a change to hMG and the stimulated cycle to obtain more oocytes, a move which yet again prompted intense debate on the relative merits of the natural or stimulated cycle in IVF. The Norfolk group had its first success in the 13th attempt in a stimulated cycle, the first American IVF baby born in December 1981.

In France, two groups were making progress in friendly competition. At the university hospital in Clamart, Frydman as clinician and Testart as biologist were focusing their research on the LH peak, and in 1981 developed an assay for the initial rise of LH in plasma (LHSIR) (9). This assay would allow the accurate prediction of the start of the LH surge, and thus more time for the organization of follicle puncture. In Sevres, a non-university hospital, the biologists Mandelbaum and Plachot and I found ourselves frustrated by the absence of a laboratory on site, and adopted a policy of transporting oocytes by thermos flask to the INSERM laboratory of the Hospital Necker, 30 minutes away by taxi. It was also at Sevres that Pez and I began tracking follicular growth by ultrasound.

Both French groups benefited from the help of veterinary researchers at INRA (Institut National de la Recherche Agronomique), one of whom, Menezo, had developed the B2 culture medium known as the "French medium."

France's first IVF babies were born at Clamart in February 1982 and at Sevres the following June. And there were now several other live births being reported from groups elsewhere—in Sweden, Finland, the Netherlands, and Germany, as well as in the United Kingdom, United States, and Australia. In Vienna, Feichtinger and Kemeter began with clomiphene cycles in the summer of 1981 and, doing their own biology, had their first live births (twins) in August 1982.

One catalyst for the surge of activity in IVF at this time was a meeting at Bourn Hall in September 1981 organized by Edwards for those groups worldwide now actively involved and reporting results—from Bourn Hall itself, Basel, Gothenburg, Kiel, Manchester, Melbourne, Norfolk, Paris, and Vienna. It was here that many of us met for the first time, and the atmosphere was warm and friendly. Comparing experiences was reassuring for everyone, and one important conclusion did emerge—a preference for stimulated cycles, which would generate more oocytes and allow a better prediction of the timing of ovulation. Now, looking back through the proceedings of that 1981 meeting and the reported discussions, I can see the following:

1. ovarian stimulation was mainly with clomiphene,
2. ultrasound was already in use (with Feichtinger) for monitoring follicular growth,
3. a concern for the effect of gas on oocyte quality during laparoscopy,
4. a concern about quality control in culture media and during laboratory processes,
5. and the conviction of Edwards that his former use of Primolut® (norethisterone) during the luteal phase of his earlier stimulated cycles would explain the failure of his first attempts at IVF; most participants at the meeting seemed to agree that, if post-aspiration progesterone values were low, a progesterone supplement would be needed during the luteal phase. Primolut, Edwards concluded, was probably an abortifacient.

If my descriptions of this first clinical phase of IVF seems to focus on just a few groups, it is because there was so little reporting of scientific data from elsewhere and because only the announcement of a pregnancy allowed some form of recognition from the scientific and lay communities. A fuller review of these pioneering days of IVF can be found in a series of articles in Human Reproduction Update by, Trounson, Dawson, Jones, Hagekamp, Nygren, Hamberger, and myself (10).

This was also, let us not forget, a period of general doubt in the scientific integrity of IVF and in its wider clinical application. In 1982, there were only 11 reported IVF births in the world, but this does not mean that the "celebrity" groups were the only ones doing IVF successfully. In many cities, there were young groups making their first attempts, and many of them traveled to the United Kingdom, United States, and Australia for their training. In the years which followed, they too would achieve their first pregnancies and live births.

1982–1992

The next decade was a time of huge progress in IVF. There was an explosion in the number of centers performing IVF in many countries, and it was also

at this time that the first discussions on the ethics of assisted reproduction began in earnest, many of which would pave the way for subsequent legislation and guidelines.

Each year saw important new clinical and scientific developments. Among the milestones were

1982: The recognition of poor and high responders to hMG, the first ultrasound-guided aspiration of follicles, and the first reports of GnRH agonist use for the downregulation of pituitary hormones in IVF (11–13)
1983: Human embryo freezing (14)
1984: The first pregnancy following gamete intrafallopian transfer (GIFT) (15)
1986: The first pregnancy following zygote intrafallopian transfer (ZIFT) (16)
1986: The first human pregnancy following oocyte freezing (17)
1988: The first report of a human pregnancy following sub-zonal insemination (18)
1989: Vitrification of human oocytes (19)
1990: The first live birth following preimplantation genetic diagnosis, the detection of aneuploidy following polar body testing, and the first description of assisted hatching (20–22)
1991: The first clinical use of GnRH antagonists for the suppression of pituitary hormones (23)
1992: Intracytoplasmic sperm injection (ICSI) (24)

ICSI would become the most successful technique introduced in the decade, thereafter applied throughout the world to overcome fertilization failure as a result of male factor or unexplained infertility. The success of ICSI would also be shown to be independent of the three basic sperm parameters, motility, morphology, and concentration.

Throughout the decade, there was a huge increase in the use of assisted reproduction and in its success. In 1986, approximately 2000 babies were born following IVF, with half of them conceived at Bourn Hall. However, by 1989, the first year of data presented in the initial world collaborative report at the Seventh World Congress of IVF in Paris in 1991, that total had increased to more than 18,000 (Table 1).

In January 1984, Seppala had sent a questionnaire to 65 individuals or groups then working in IVF which had produced data on 10,028 cycles. Success rates according to the type of ovarian stimulation is shown in Table 2, and according to the number of embryos transferred in Table 3. In 1988, I reported a similar collaborative study at the Sixth World Congress of IVF in Melbourne which showed that, of 2342 pregnancies in the database, 24.8% were spontaneously lost and 5.2% were ectopic (Tables 4 and 5).

Table 1 In Vitro Fertilization: 1989 General Data

	FR	USA	UK	Aus/ NZ	DE	Scand	BE	JP	CA	ES
Clinics reported	115	180		24	40	33	14	124	14	
Clinics participating	50	161	35	23	37	25	14	67	10	10
Studied cycles	15,880	18,211	10,489	9345	8385	6245	4578	3726	3180	
OPU cycles	15,725	15,392	8514	7356	5759	5379	3750	3438	2724	1247
Transfer cycles	10,531 (+999)	13,523	6553	6261	4365	4581 (+100)	3040	2571 (+93)	2233	1158
Clinical pregnancies	2526 (+142)	2811	1354	1040	900	997 (+21)	741	421 (+5)	391	244
Deliveries	1893[a]	2146	982	755	646	705[a]		306[a]	264	194
Babies including stillborn	2531[a]	2929		964		926[a]		391	286	232
Total babies reported since start	11,127	11,015		4595	3275	1864	2428	552	1337	
Abnormal babies	181					27	18		22	

[a]Including frozen-thawed transfers (numbers in parentheses).
FR; France, USA; United States of America, UK; United Kingdom, AU; Australia, KR; Korea, CZ; Czechoslovakia, GR; Greece, Yug; Yugoslavia, NL; Netherlands,
Abbreviation: IVF, in vitro fertilization.
Source: From Ref. 35.

Table 2 Type of Ovarian Stimulation and Number of Pregnancies Achieved

Stimulation	No. of reporting teams	No. of pregnancies/ No. of cycles	Success (%) per cycle
None; natural cycle	7	41/352	11.6
Clomiphene/hCG	44	256/3083	8.3
Clomiphene/hMG/hCG	50	377/3847	9.8
Clomiphene[a]	14	167/980	17.0
Clomiphene/hMG[a]	7	53/340	15.6
hMG/hCG	41	235/1591	14.8

[a]Spontaneous LH surge.
Abbreviations: hMG, human menopausal gonadotropin; hCG, human chorionic gonadotropin.
Source: From Ref. 36.

KR	CZ	GR	Yug	NL	SG	CN	BR	IN	PT	EG	TR	IE	Total	%OPU
5	5	8	7	1	6	7	3	9	3	4	1	1	≥649	
3	5	6	2	1	6	4	3	4	2	1	1	1	469	
1456	1456	1232		769	696	617	504	383	271	243	66		>87,732	
1240	1234	1000	857	656	618	509	455	322	222	168	66	34	76,030	100
1019 (+41)	524	835 (+21)	655	531 (+49)	483 (+60)	358	383	267 (+18)	178	139	65	29	60,282	79.3
191 (+2)	51	156 (+1)	89	140 (+7)	110 (+11)	94	85	51	51	27	8	2	12,480	16.4
137[a]	32	100	61	112[a]	95[a]		68	33[a]	40	20	6	0	>8595	12.0
122[a]	34	98	72	162[a]	137[a]		90	50[a]	62	31	8		>9125	
151	55	206	153	399	139	202	171	109	117	105	13		>38,013	
3	0	4	1	15	0	2		2	3	0	0		>278	1.5

NZ; New Zealand, DE; Germany, Scand; Scandinavia, BE; Belgium, JP; Japan, CA; Canada, ES; Spain, SG; Singapore, CN; China, BR; Brazil, IN; India, PT; Portugal, EG; Egypt, TR; Turkey, IE; Ireland.

It seems worthwhile to pause here and reflect on our main concerns during this decade of such great progress in reproductive medicine. First, our main scientific efforts were concentrated on fertility itself, whether to prevent pregnancy with contraception or to facilitate it with assisted reproduction. In IVF, we were looking for ways to increase the number of oocytes available for fertilization but to decrease the number of sperm cells necessary to achieve it (as it was by now quite clear that the failure of fertilization was often the result of a low concentration of motile sperms). At the same time, we were also searching for ways—as reflected in the techniques of zona drilling or partial zona dissection, or indeed in GIFT or ZIFT—to bring gametes closer together in time and space, and break through the physiological barriers of the oocyte.

However, the indications for IVF were not yet changing in any major way—and would not until the introduction of ICSI opened a door to the treatment of male infertility. From the beginning, IVF had remained indicated mainly for the treatment of tubal infertility as a result of blocked or damaged Fallopian tubes. Thus, there was a lively debate in the early

Table 3 Clinical Pregnancies Relative to Number of Embryos Replaced

No. of embryos replaced	No. of pregnancies/ No. of replacement cycles	Success rate (%)
One embryo	317/3321	9.5
Two embryos	366/2514	14.6
Three embryos	259/1340	19.3
Four or more embryos	197/818	24.1

Source: From Ref. 36.

1980s following developments in microsurgery on how tubal blockage might best be treated; the microsurgeons were insistent that their new surgical techniques were potentially more effective than IVF. However, IVF quickly won that debate by gradually extending its indications far beyond the range of surgery, first into tubal infertility with patent but diseased tubes, and then into polycystic ovary disease and other idiopathic conditions. By the time the indications had been stretched to male infertility following the introduction of ICSI in the early 1990s, the debate between assisted reproduction and surgery was long over. Today, ICSI accounts for around 40% of all the indications for assisted reproductive technology (ART).

Table 4 Features of the Population Under Study: 2342 Pregnancies in Women of Mean Age of 33 Yr

(%)	Indications for IVF	Type of ovarian stimulation	Oocyte collection
Tubal	67.9		
Idiopathic	11.0		
Male infertility	3.5		
Other	16.7		
Clomiphene		3.9	
hMG		20.8	
FSH		3.4	
CC/hMG		62.7	
hMG/FSH		7.4	
Other		1.8	
By ultrasound			22.6
By laparoscopy			77.4

Abbreviations: IVF, in vitro fertilization; hMG, human menopausal gonadotropin; FSH, follicle stimulating hormone.
Source: From Ref. 37.

Table 5 Incidence of the Different Pregnancy Outcomes (2329 cases)

Early abortions	24.5 (%)
Late abortions	1.5 (%)
Ectopic pregnancies	5.2 (%)
Births	62.5 (%)
Ongoing pregnancies	7.5 (%)

Source: From Ref. 37.

There was also another trend evident throughout this important decade. It was clear by the early 1980s that ovarian stimulation with gonadotrophins would allow the collection of more oocytes for fertilization and more embryos for transfer. However, with no limit on the number of embryos transferred and clinics anxious to increase their success rates, the number (and proportion) of multiple pregnancies was seen to increase in parallel to the wider use of ovarian stimulation. Multiple pregnancies would become one of the real issues of ART, both in terms of health and cost.

There were also major changes introduced in the technicalities of IVF: egg collection via laparoscopy was almost totally replaced by the far less invasive, ultrasound-guided transvaginal route; and there were also at this time great improvements made in the composition of culture media and in the processes of laboratory quality control.

And for the patient, what was the benefit of these developments? There were still those who argued that IVF was an inefficient procedure, with success rates improving only marginally with each scientific advance. However, in the adoption of embryo freezing, patients had more opportunity for embryo transfer from a single stimulation, and with it a much greater chance of success. Even in 1991, when I presented results from the world collaborative report in Paris, I reported a 20.7% pregnancy rate from fresh embryo transfers, and 13.7% from frozen (25). And it is also worth noting that throughout the decade the application of gamete donation developed remarkably such that with the introduction of oocyte and embryo donation, even those women with premature ovarian failure now had the chance to have their own babies, even if from a donated egg.

There were other developments too, outside the laboratory and clinic. Even before the birth of Brown, both Edwards and Steptoe were in favor of ethical discussion about IVF. The British government was the first to appoint a commission of enquiry into all forms of assisted conception in 1982 (under the chairmanship of Warnock), which reported 2 years later with a list of recommendations, including a statutory licensing authority. The United Kingdom was the first country to introduce legislation and regulation to assisted reproduction.

What was, clear, however, with legislation or not, was that each successive discovery in IVF stimulated some ethical discussion, and the press, politicians, theologians, and medical groups all raised concerns about such rapid progress into new forms of human conception. These discussions are clearly set out in a chapter titled "The History and Ethics of Assisted Conception" in a 1995 textbook on ART by Edwards and Brody (26).

There was a broad disparity in how different countries adopted different moral positions with respect to the new reproductive technologies. In some countries, politicians and representatives of society were the final arbiters, whereas in others clinicians and scientists were left to define their own codes of practice. In 1999, Jones and I, on behalf of the IFFS, published a review of the guidelines and legislation in place in 38 countries, and could not find even two of those countries sharing the same legal positions (27). Indeed, even in the same geographical group of countries, there were significant differences: in Australia, there were even different laws on the two sides of a state border; in France, patients were forced to go to Belgium for pre implantation diagnosis (PGD) or oocyte donation; in the Nordic countries, Iceland and Finland had no legislation in place, whereas Denmark, Norway, and Sweden each had contradictory legislation with respect to gamete freezing and donation.

These paradoxes were essentially created by politicians and healthcare regulators and resulted mainly in sizable traffic of infertile couples seeking treatment beyond their own legislative borders, thereby initiating the "import and export" of ART. Even if the legislation has changed over the years, the problem of "reproductive tourism" has not.

Thus, even from the pioneering days of IVF, clinicians and biologists have shared a concern for the moral responsibility of providing IVF that was not always the same as the legislators'. Both of the leading scientific societies in reproductive medicine have continued to encourage discussions on its ethics, with debate, taskforce review, and publications. However, 25 years after the birth of Brown, there remain different opinions adopted by ethicists on the status of the human embryo which find their way into the contradictory laws which still persist in many countries.

1992–THE PRESENT

Although the outcome of ART procedures appears to have shown a constant, if slow, improvement throughout the 1990s, it has always been difficult to record precise results and compare them from one year to the next or from one country to another. Many registries have been set up, but most submissions and results were not audited, and wide methodological discrepancies remain between one registry and the next. Nevertheless, most reports show that, although the mean delivery per embryo transfer increased from 22% in 1995 to 31% in 2000, some individual groups achieved published rates of 40% or more.

Any progress in these results appears to have been modest in recent years, but the problems emerging in the 1990s have remained: a high rate of pregnancy loss (25%) and a high incidence of multiple pregnancies (25–40%) (28). The result is that today the avoidance of multiple pregnancies after ART has become one if its main goals, with legislation now in place in some countries to limit the number of embryos transferred to two or even one.

In recent years, there have also been several studies showing that even singleton children born after ICSI or IVF have an increased risk of malformation or some developmental abnormality. The possible source of these risks has been associated with ovarian stimulation, laboratory procedures, or infertility itself. Prospective studies designed to identify the etiology of these problems are needed, even though most studies so far suggest that the causes of the infertility itself are the main associations with risk.

Since the introduction of ICSI in the early 1990s, ART has continued to pass significant milestones:

1994: Pregnancy following fertilization with sperm cells retrieved from the testes or epidydimis, and in vitro maturation (29,30)
1997: Blastocyst transfer (31)
1998: Mitochondrial transfer between oocytes (32)
2001: Single embryo transfer (33)
2004: First pregnancy following implantation of an embryo obtained from frozen ovarian tissue (34)

In the 21st century, it is clear how much the new reproductive technologies have allowed and initiated advances in genetics. The ability to transfer embryos screened for chromosomal and single gene defects has reduced the risk of many inherited diseases, while immunoassay technology has provided detailed insight into the cellular processes involved in gene expression. However, such developments—as well as the publicly perceived ability to select embryos for their sex and genetic characteristics—have raised fears of "designer babies" and a move towards some kind of eugenics under pressure of parents.

Since the birth of Dolly, the sheep in Scotland in 1996, the issue of reproductive or therapeutic cloning has been exposed. In therapeutics, the transplantation of human embryonic stem cells now holds great promise for the treatment of diseases such as Parkinson's or diabetes, whereas developments in stem cell biology will lead to a better understanding of infertility, implantation failure, genomic imprinting, and meiosis. The gynecologist could take part in that research, but for now the initiative lies with the scientists and the active work of the geneticist.

REFERENCES

1. Chang MC. Fertilization and normal development of follicular oocytes in the rabbit. Science 1955; 121:867.

2. Chang MC. Fertilization of rabbit ova in vitro. Nature 1959; 184(suppl 7):466.
3. Edwards RG. Maturation in vitro of human ovarian oocytes. Lancet 1965; 2:926.
4. Pincus G. The Control of Fertility. New York: Academic Press, 1965.
5. Borth R, Lunenfeld B, de Watteville H. Activite gonadotrope d'un extrait d'urines de femmes en menopause. Experimentia 1954; 10:266.
6. Gemzell CA, Diczfalusy E, Tillinger K. Clinical effect of human pituitary follicle-stimulating hormone (FSH). J Clin Endocrinol Metab 1958; 18:1333.
7. Klein R, Palmer R. Technique of sampling human ova by follicular puncture under celioscopy. CR Seances Soc Biol Fil 1961; 155:1919.
8. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978; 2:366.
9. Testart J, Frydman R, Feinstein MC, et al. Interpretation of plasma luteinizing hormone assay for the collection of mature oocytes from women: definition of a luteinizing hormone surge-initiating rise. Fertil Steril 1981; 36:50.
10. Cohen J, Trounson A, Dawson K, et al. The early days of IVF outside the UK. Hum Reprod Update 2005; 11:439.
11. Jones HW Jr., Jones GS, Andrews MC, et al. The program for in vitro fertilization at Norfolk. Fertil Steril 1982; 38:14.
12. Lenz S, Lauritsen JG. Ultrasonically guided percutaneous aspiration of human follicles under local anesthesia: a new method of collecting oocytes for in vitro fertilization. Fertil Steril 1982; 38:673.
13. Fleming R, Adam AH, Barlow DH, et al. A new systematic treatment for infertile women with abnormal hormone profiles. Br J Obstet Gynaecol 1982; 89:80.
14. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. Nature 1983; 305:707.
15. Asch RH, Ellsworth LR, Balmaceda JP, et al. Birth following gamete intrafallopian transfer. Lancet 1985; 2:163.
16. Devroey P, Braeckmans P, Smitz J, et al. Pregnancy after translaparoscopic zygote intrafallopian transfer in a patient with sperm antibodies. Lancet 1986; 1:1329.
17. Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986; 1:884.
18. Ng SC, Bongso A, Ratnam SS, et al. Pregnancy after transfer of sperm under zona. Lancet 1988; 2:790.
19. Pensis M, Loumaye E, Psalti I. Screening of conditions for rapid freezing of human oocytes: preliminary study toward their cryopreservation. Fertil Steril 1989; 52:787.
20. Handyside AH, Kontogianni EH, Hardy K, et al. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 1990; 344:768.
21. Verlinsky Y, Ginsberg N, Lifchez A, et al. Analysis of the first polar body: preconception genetic diagnosis. Hum Reprod 1990; 5:826.
22. Cohen J, Elsner C, Kort H, et al. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod 1990; 5:7.
23. Fleming R. Gonadotrophin-releasing hormone agonist and estrogen–progestogen replacement therapy. Am J Obstet Gynecol 1991; 165:1156.

24. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992; 340:17.
25. Cohen J, Testart J, Mandelbaum J, Plachot M, Frydman R. World Collaborative Report 1989, distributed at the 7th World Congress on IVF, Paris, Cotten 30 June 1991.
26. Edwards RG, Brody SA. Principles and Practice of Assisted Human Reproduction. Philadelphia: Saunders Company, 1995.
27. Jones HW Jr., Cohen J. Surveillance 1998. Fertil Steril 1999; 71:796.
28. Nyboe-Andersen A, Gianaroli L, Nygren KG. The European IVF Monitoring Programme (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). Assisted Reproductive Technology in Europe, 2000. Results generated from European registers by ESHRE. Hum Reprod 2004; 19:490.
29. Silber SJ, Nagy ZP, Liu J, et al. Conventional in vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. Hum Reprod 1994; 9:1705.
30. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil Steril 1994; 62:353.
31. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Hum Reprod Update 1997; 3:367.
32. Cohen J, Scott R, Schimmel T, et al. Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. Lancet 1997; 350:286.
33. Vilska S, Tiitinen A, Hyden-Granskog C, et al. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. Hum Reprod 1999; 14:2392.
34. Oktay K, Tilly J. Live birth after cryopreserved ovarian tissue autotransplantation. Lancet 2004; 364:2091.
35. Cohen J. In Vitro Fertilization Congress, Paris, 1991.
36. Seppala M. The world collaborative report on in vitro fertilization and embryo replacement: current state of the art in January 1984. In: Seppala M, Edwards RG, eds. In Vitro Fertilization and Embryo Transfer. New York: Annals of the New York Academy of Sciences, 1985:558.
37. Cohen J, Mayaux MJ. Guihard-Moscato, Pregnancy outcomes after in vitro fertilization: a collaborative study on 2342 pregnancies. In Vitro Fertilization and Embryo Transfer. Annals of the New York Academy of Sciences New York1986:1.

2

Evaluation and Preparation of the Infertile Couple for In Vitro Fertilization

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Thorough evaluation of the infertile couple before in vitro fertilization (IVF) is critical in achieving the best outcomes and avoiding complications. Most IVF centers organize the evaluation by using a checklist that the nurse coordinator and physician assure is complete before proceeding with the cycle.

DAY 3 FOLLICLE-STIMULATING HORMONE

A level exceeding 25 mIU/ml (about 12 mIU/ml using current assays) has been correlated with a very low chance of pregnancy (1). More recent studies have shown that mild elevations in women below 40 yr of age predict a more modest reduction in the pregnancy rate, whereas an elevated level carries much more meaning in older women. Sometimes, particularly in older women, follicular maturation is very rapid and the follicle-stimulating hormone (FSH) can already be decreasing by day 3. Therefore, the level of estradiol (E_2) should also be measured. The impact of an increased day 3 E_2 level (over 70–80 pg/ml) in the presence of a normal FSH concentration is unclear, but using gonadotropin releasing hormone (GnRH) agonist and assisted hatching, an elevated E_2 level correlates with increased cycle cancellation but not with a reduced pregnancy rate (2). An elevated day 3 E_2 has less importance in young women. We also use the FSH assay to predict the

optimal level of stimulation, because the ovarian response has been shown to vary inversely with the FSH level (1). For women with an FSH level over 10 mIU/ml, we generally choose a low responder protocol.

FSH assays vary considerably in their normal ranges. Ideally, if switching from the Leeco Diagnostics, Southfield, Michigan Company, (now Binax, Inc., Scarborough, Maine) assay on which older research was based (1), a series of samples should be run in parallel using both methods, so that the new assay levels can be interpreted appropriately. In our case, when switching to the Immulite (DPC) system, a level of 12 mIU/ml corresponded to 25 mIU/ml in the BINAX system. In the absence of such direct comparison, one can use the College of American Pathologists survey booklet, which gives mean levels for all labs using each kit and standard sera.

FSH levels also vary from cycle to cycle. A consistently elevated FSH predicts a poorer prognosis than a single elevated level with others being normal. The quality of ovarian stimulation is not improved when IVF is done in a cycle with a more normal FSH level (3). There is an agreement that women with a single elevated FSH level have a high cancellation rate, but studies conflict regarding the extent of reduction of pregnancy outcome (4,5). FSH levels are similar on days 2, 3, and 4. Women with premenstrual spotting should be advised to count the first day of full flow as day 1.

ANTRAL FOLLICLE COUNT

It is the antral follicles that respond to stimulation. With a high-quality transvaginal ultrasound scan, these can be accurately counted. Follicle count decreases with age in normal women (6). In women with 5–10 follicles per side, one expects a normal response to stimulation. With more than 10 per side [polycystic ovary (PCO)-like], a lower level of stimulation should be chosen than otherwise would be used based on weight and FSH level. A low follicle count (fewer than 5 or 6 in total) predicts a lower prognosis (7,8) and should prompt a higher level of stimulation. Total follicle count correlates positively with the number of oocytes retrieved and negatively with day 3 FSH and ampoules of gonadotrophins, with fewer than 10 total follicles predicting an increased chance of cancellation (9). By multivariate analysis, antral follicle count was found to be the best single predictor of ovarian response and therefore prognosis, with FSH having a small additive effect (10). As the outcome of IVF is very low in women above 40 years of age who develop fewer than three follicles with stimulation (11), a low resting follicle count can be used together with other data (age, day 3 FSH, duration of infertility) to suggest egg donation as a better option.

CLOMIPHENE CITRATE CHALLENGE TEST

The clomiphene citrate challenge test (CCCT) has been used to identify patients with a low prognosis and low ovarian reserve who have a normal

day 3 FSH level (12). CC is taken at 100 mg/day from days 5 to 9. The day 10 FSH level should be less than 10–12 mIU/ml. In normal women, although FSH is stimulated by CC, the rising E_2 brings it back into the normal range. In women with low ovarian reserve, the pituitary responds with a more prominent FSH rise which is not suppressed as promptly by the rising E_2 level. Clearly there is a group of women with a normal day 3 FSH with an abnormal CCCT who have a reduced prognosis, but in the usual instance the couple will choose to go ahead regardless, and other information such as the antral follicle count will be sufficient to plan the ovarian stimulation. A recent study looking at various markers of ovarian reserve failed to find any clear additional predictive value for poor ovarian response in addition to FSH and antral follicle count (13).

POLYCYSTIC OVARIAN DISEASE

Women with PCO produce more follicles with stimulation. More oocytes are retrieved, having a lower fertilization rate. The pregnancy rate is as good as other women having IVF. Provided a GnRH agonist is used, the miscarriage rate is normal (14).

Metformin, which lowers circulating insulin levels and the ovarian production of androgens, has been found to reduce the follicular and estradiol response to stimulation and to increase the number of mature oocytes and embryo quality and the pregnancy rate in clomiphene-resistant women with PCO (15). In a subsequent study of unselected PCO women, the success rate was significantly increased only in normal weight women (16). As insulin resistance is more common in women who are clomiphene-resistant, that clinical group and insulin resistance may be particularly strong indications for this adjunctive treatment. PCO women on metformin who are coasted have lower peak estradiol levels and fewer days of coasting (17). As insulin is one of the main factors that stimulate vascular endothelial growth factor production by luteinized granulosa cells, and metformin decreases ovarian response and circulating insulin levels, metformin may be an important aid in reducing ovarian hyperstimulation syndrome in these women.

SEMEN ANALYSIS WITH WHITE BLOOD CELLS STAIN AND CULTURE

A semen analysis is done before the cycle to assure that semen quality is not at a nadir for that individual due to recent factors such as stress or a febrile illness. In general, IVF is preferred with reduced semen quality, as gamete intrafallopian transfer (GIFT) has been less successful than with normal sperm, and IVF allows confirmation of whether fertilization occurred. Pyospermia can reduce sperm function (18). We attempt to clear pyospermia before proceeding to IVF. Frequent ejaculation may augment the action

of antibiotics. Semen culture is probably worthwhile as a routine, in order to prevent the occasional contamination of the culture which will otherwise occur.

STRICT MORPHOLOGY

Cases of unexplained failure of fertilization have been found to be due to unrecognized subtle abnormalities of sperm structure. When strict morphology shows 4% or fewer normal sperm, the chance of failed fertilization is high. Insemination with a larger sperm number raises the fertilization rate to almost normal but the percentages of implantation and ongoing pregnancy/delivery are reduced by 40–50% (19), whereas intracytoplasmic sperm injection (ICSI) has been as successful as with other infertility factors (20). These findings suggest an embryotoxic effect of a high concentration of these very abnormal sperm which can be avoided by achieving fertilization with ICSI. In some cases, sperm morphology improves with observation or treatment with antioxidants. Sperm morphology may be impaired in smokers and may be improved by giving vitamin C, 1.0 g daily.

ANTISPERM ANTIBODIES

Antisperm antibodies (ASAs) in the female can impair or prevent fertilization if the female partner's serum is used in the insemination medium (21). Routine or selective use of fetal cord serum, human serum albumen, or donor serum will prevent this problem. As ASAs are also present in follicular fluid, we do extra washes of the cumulus and add an increased number of sperm. Although GIFT has been just as successful in women with as without ASAs (22), most women with high levels have probably been advised to have IVF. Female ASAs are more common when testing is done with her partner's sperm, suggesting antibody production to husband-specific antigens as well as non-specific sperm antigens. We currently test the husband's sperm against his wife's and against a negative control serum using the immunobead test.

Male ASAs may result from infection, trauma, or surgery, or may occur without any positive history. With greater than 70% IgG and IgA binding, there is a high chance of fertilization failure with routine insemination of the oocytes. ICSI is usually advised with high ASA levels.

SPERM PENETRATION ASSAY

It has been controversial whether the sperm penetration assay (SPA) is helpful, but one large study showed a very high predictive value of a 0% penetration rate with failed fertilization using a standard insemination number (23). Alternative methods of sperm preparation can improve both the SPA (24,25) and the fertilization rate (e.g., test yolk buffer and follicular fluid). If we have a couple who had their SPA done with test yolk buffer (TYB),

we always use TYB for their IVF. Otherwise, one could have failed fertilization in an individual whose sperm only develop adequate capacity with TYB. As we have found consistent good results with the SPA with TYB, we now routinely use a 2-hr incubation with TYB for the IVF cycle but seldom do the SPA.

SPERM CHROMATIN STRUCTURE ASSAY

Fragmented DNA can be an unrecognized cause of infertility. This can now be determined clinically by flow cytometry sperm chromatin structure assay (SCSA). Although there is some correlation of abnormal sperm parameters with the SCSA (26), a high level of DNA fragmentation may occur with normal or mildly impaired morphology. In a recent study, antioxidant therapy was shown to improve the SCSA score. The impact of a high SCSA can be lessened by density centrifugation. A 450% improvement in nuclear integrity has been achieved with a 45–90% PureSperm® (Nidacon, Gothenburg, Sweden) gradient (26). Retrieval of testicular sperm may be an option for men with continuing high DNA fragmentation (27). In the same individuals, the level of fragmentation in testicular sperm averaged 5%, compared to 24% in the ejaculate. As there is a correlation with motility and morphology, choosing the most active and morphologically normal sperm for ICSI will also choose the sperm most likely to have intact DNA.

CHLAMYDIA

A number of reports have found a negative relationship of positive chlamydia antibodies to successful pregnancy (28,29). In one study, a significantly higher miscarriage rate was noted (30). This may be due to chronic endometrial infection or permanent effects of prior infection. Unfortunately, the endometrium can be positive with negative cervical cultures (31). In fact, in one study of 28 infertile couples with negative cultures or DNA probe assays, 40% were found to have active chlamydia infection by PCR. Because of these findings, we have elected to routinely treat both partners with a 10-day course of doxycycline. This may also eradicate ureaplasma and unrecognized semen or pelvic infections which could also compromise the outcome.

TRIAL TRANSFER

We have always done a rehearsal of the transfer with measurement and mapping of the endometrial canal. A controlled study has documented a significant increase in the pregnancy rate with this having been done with a reduced incidence of difficult transfers (32). It is helpful to do this under ultrasound guidance, in order to define the optimal conditions for

embryo transfer. Cervical dilation has been shown to reduce the incidence of difficult transfers (33). Hysteroscopy has been used in very difficult cases to shave away ridges or cysts obstructing passage of the catheter (34).

UTERINE AND TUBAL ABNORMALITIES

The success rate with GIFT in women with tubal disease is not greater than with IVF, and the risk of tubal pregnancy is higher. Therefore, IVF is most appropriate with significant tubal abnormalities. We examine the uterine cavity with ultrasound before and during ovarian stimulation. Significant polyps or myomata are often easily visualized. A sono-hysterogram or hysteroscopy should be done if there is a further question of uterine disease. A recent randomized, controlled study has shown a higher pregnancy rate following hysteroscopic excision of small (mean 16 mm) polyps, underlining the importance of a thorough evaluation of the uterine cavity (35). Generally, a uterine septum should be incised before going on to IVF because of the higher risk of spontaneous abortion. Several recent studies have found approximately a 50% reduction in the rate of delivery in women with a hydrosalpinx compared with women with tubal disease without a hydrosalpinx (36). The success rate increases to normal after tubal repair or salpingectomy (37). Endometrial integrin is reduced in many patients with hydrosalpinx and reverts to a normal pattern after salpingectomy (38). Occlusion of the proximal tube seems to be equally efficacious (39). Spontaneous pregnancy can occur when a unilateral hydrosalpinx is removed (40) or repaired. It has been suggested that only hydrosalpinges which are visible on transvaginal ultrasound should be removed (41). However, hydrosalpinges enlarge during stimulation (42) and may become visible only during the IVF cycle. A recent randomized study showed increased fecundity following excision of polyps compared to only biopsy. Other studies have suggested that the polyp excision itself may enhance implantation from the healing process. A randomized study showed that a biopsy done in the cycle immediately preceding IVF was associated with increased implantation.

HIV AND HEPATITIS

Most programs screen for HIV and hepatitis for safety of personnel. It would also be tragic to expend the amount of effort required to achieve an IVF pregnancy only to have the offspring at risk for a potentially fatal disease. With hepatitis B, the female partner should be immunized. With HIV, sperm separation and ICSI is being used by some programs to avoid transmission of the virus.

ENDOMETRIOSIS

Some studies have shown reduced rates of implantation with severe or extensive endometriosis, and unexplained failure of fertilization has been

reported in some women with endometriomas. A recent meta-analysis showed an odds ratio of successful pregnancy with IVF of 0.56 in women with endometriosis (43). Even in the presence of mild endometriosis, quantitative defects of the secretory response of endometrial glandular cells and other endometrial abnormalities have been described. Any endometrioma fluid should be kept separate from aspirates containing oocytes, and aspirating needles and pipettes should be changed. Two randomized studies have shown that a 3–6-mo course of GnRH agonist leading directly into IVF is associated with an increased pregnancy rate in women with stage III and IV endometriosis (44,45).

DIETHYLSTILBESTER (DES) EXPOSURE

Viable pregnancy is reduced by about 50% with a history of DES exposure (46). Outcome is particularly poor with constrictions or a T-shaped cavity but is normal when the cavity is merely small.

UTERINE FIBROIDS

Submucus fibroids markedly reduce the pregnancy rate with IVF (47). Studies have been conflicting regarding the role of intramural myomas, with some studies showing a significant reduction of outcome (47–49) and others not showing an effect (50–52). With relatively small studies, the statistical power is such that some studies may not detect a significant impact. It is likely that intramural myomas reduce implantation, but the effect is probably small unless the uterine cavity is distorted. Very large numbers would be required to accurately quantify such an effect. At the present time, we advise excision if they are large or distort the cavity.

SEXUAL DYSFUNCTION

Rarely, anxiety can lead to total inability to provide a semen specimen on the day of retrieval. Frozen husband's sperm has been found to yield a fairly normal rate of fertilization provided an increased number of sperm is added (53). In our detailed instructions to patients, we state in bold print: "If you anticipate any problems providing a semen specimen on the day of retrieval, please tell us. We can arrange for you to freeze a specimen as a back-up." A supply of Viagra should be available for any male having difficulty collecting a specimen.

PERSONAL HABITS

Meta-analysis of studies on the effect of smoking on IVF conception rate revealed an odds ratio of 0.54 (95% CL 0.34–0.75) (54). Smoking also

increases the rate of spontaneous abortion. We strongly recommend that all women stop smoking before having IVF.

A study of caffeine use found that intake of 2 mg (equivalent to one cup of decaf coffee) or less was associated with the highest pregnancy rate with IVF (55). Although not confirmed by other studies, avoiding caffeine is a simple measure to undertake.

Obesity correlates negatively with implantation (56). Therefore, weight loss may improve IVF results.

Studies on alcohol and fertility are conflicting, with some showing impaired fertility with small amounts of alcohol (57), whereas in one study, wine drinkers had a shorter time to conception (58).

PSYCHO-SOCIAL ASPECTS

Stress, anxiety, and depression have been linked to lower IVF outcomes (59–61), and psychological intervention improves the chance of success (62). Paying attention to these factors will also improve interactions of patients and staff, and will help adjustment to the stresses of child rearing. Multiple pregnancies have been shown to cause considerable personal and marital stress. Early intervention may enhance the long-term well-being of these families. Couples should plan their IVF cycle for a time of lowest possible stress.

GENERAL HEALTH

Regular health screening such as pap smear or mammography can be easily forgotten during an extended course of fertility treatments. All appropriate health screening should be completed before embarking on pregnancy to avoid a significant health issue arising during pregnancy. For all egg donation recipients, we do a more extensive evaluation including a stress electrocardiogram, chemistry panel, and chest X-ray.

REFERENCES

1. Muasher SE, Oehninger S, Simonetti S, et al. The value of basal and or stimulated serum gonadotropin levels in prediction of stimulation response and in vitro fertilization outcome. Fertil Steril 1998; 50:298.
2. Frattarelli JL, Bergh PA, Drews MR, et al. Evaluation of basal estradiol levels in assisted reproductive technology cycles. Fertil Steril 2000; 74:518.
3. Scott RT, Hofmann GE, Oehninger S, et al. Intercycle variability of day 3 follicle-stimulating hormone levels and its effect on stimulation quality in in vitro fertilization. Fertil Steril 1990; 53:297.
4. Martin JSB, Nisker JA, Tummon JS, et al. Future in vitro fertilization pregnancy potential of women with variably elevated day 3 follicle-stimulating hormone levels. Fertil Steril 1996; 5:1238.

5. Lass A, Gerrard A, Abusheikha N, et al. IVF performance of women who have fluctuating early follicular FSH levels. J Assist Reprod Genet 2000; 17:566.
6. Scheffer GJ, Broekmans FJM, Dorland M, et al. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. Fertil Steril 1999; 72:845.
7. Huang F-J, Chang S-Y, Tsai M-Y, et al. Determination of the efficiency of controlled ovarian hyperstimulation in the gonadotropin-releasing hormone agonist-suppression cycle using the initial follicle count during gonadotropin stimulation. J Assist Reprod Genet 2001;18:91.
8. Nahum R, Shifren JL, Chang Y, et al. Antral follicle assessment as a tool for predicting outcome in IVF—is it a better predictor than age and FSH? J Assist Reprod Genet 2001; 18:151.
9. Frattarelli JL, Lauria-Costa DF, Miller BT. Basal antral follicle number and mean ovarian diameter predict cycle cancellation and ovarian responsiveness in assisted reproductive technology cycles. Fertil Steril 2000; 74:512.
10. Bancsi LF, Broekmans FJ, Eijlmans MJ, et al. Predictors of poor ovarian response in in vitro fertilization: a prospective study comparing basal markers of ovarian reserve. Fertil Steril 2002; 77:328.
11. Biljan MM, Buckett WM, Dean N, et al. The outcome of IVF-embryo transfer treatment in patients who develop three follicles or less. Hum Reprod 2000; 15:2140.
12. Scott RT, Leonardi MR, Hoffman GE, et al. A prospective evaluation of clomiphene citrate challenge test screening in the general infertility population. Obstet Gynecol 1993; 82:539.
13. Hendriks DJ, Broekmans FJM, Bancsi LFJMM, et al. Repeated clomiphene citrate challenge testing in the prediction of outcome in IVF: a comparison with basal markers for ovarian reserve. Hum Reprod 2004; 20:163.
14. Balen AH, Tan S-L, MacDougall J, et al. Miscarriage rates following in vitro fertilization are increased in women with polycystic ovaries and reduced by pituitary desensitization with buserelin. Hum Reprod 1993; 8:959.
15. Stadtmauer LA, Torna SM, Riehl RM, et al. Metformin treatment of patients with polycystic ovary syndrome undergoing in vitro fertilization improves outcomes and is associated with modulation of the insulin-like growth factors. Fertil Steril 2001; 75:505.
16. Kjotrod SB, von During V, Carlsen SM, et al. Metformin treatment before IVF/ICSI in women with polycystic ovary syndrome; a prospective, randomized, double blind study. Hum Reprod 2004; 19:1315.
17. Stadtmauer LA, Toma SK, Riehl RM, et al. Impact of metformin therapy on ovarian stimulation and outcome in ‘coasted’ patients with polycystic ovary syndrome undergoing in vitro fertilization. Reprod Biomed Online 2002; 5:112.
18. Wolff H, Politch JA, Martinez A, et al. Leukocytospermia is associated with poor semen quality. Fertil Steril 1990; 53:528.
19. Grow DR, Oehninger S, Seltman HJ, et al. Sperm morphology as diagnosed by strict criteria: probing the impact of teratozoospermia on fertilization rate and pregnancy outcome in a large in vitro fertilization population. Fertil Steril 1994; 62:559.
20. Oehninger S, Kruger TF, Simon T, et al. A comparative analysis of embryo implantation potential in patients with severe teratozoospermia undergoing in

vitro fertilization with a high insemination number of intracytoplasmic sperm injection. Hum Reprod 1996; 11:1086.
21. Mandelbaum SL, Diamond MP, De Cherney AH. Relationship of antisperm antibodies to oocyte fertilization in in vitro fertilization–embryo transfer. Fertil Steril 1987; 47:644.
22. In vitro fertilization–embryo transfer (IVF–ET) in the United States: 1989 results from the IVF-ET Registry. Fertil Steril 1991; 55:14.
23. Margalioth EJ, Navot D, Laufer N, et al. Correlation between the zona-free hamster egg sperm penetration assay and human in vitro fertilization. Fertil Steril 1986; 45:665.
24. Katayama KP, Stehlik E, Roesler M, et al. Treatment of human spermatozoa with an egg yolk medium can enhance the outcome of in vitro fertilization. Fertil Steril 1989; 52:1077.
25. Ghetler Y, Ben-Nun I, Kaneti H, et al. Effect of sperm preincubation with follicular fluid on the fertilization rate in human in vitro. Fertil Steril 1990; 54:944.
26. Tomlinson MJ, Moffatt O, Manicardi GC, et al. Inter-relationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. Hum Reprod 2001; 16:2160.
27. Greco E, Scarselli F, Iacobelli M, et al. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod 2004; 20:226.
28. Rowland GF, Forsey T, Moss TR, et al. Failure of in vitro fertilization and embryo replacement following infection with chlamydia trachomatous. J In Vitro Fert Embryo Transfer 1985; 2:151.
29. Lunenfeld E, Shapiro BS, Sarov B, et al. The association between chlamydial-specific IgG and IgA antibodies and pregnancy outcome in an in vitro fertilization program. J In Vitro Fert Embryo Transfer 1989; 6:222.
30. Licciardo F, Grifo JA, Rosenwaks Z, et al. Relation between antibodies to chlamydia trachomatous and spontaneous abortion following in vitro fertilization. J Assist Reprod Genet 1992; 9:207.
31. Shepard MK, Jones RB. Recovery of chlamydia trachomatous from endometrial and fallopian tube biopsies in women with infertility of tubal origin. Fertil Steril 1989; 52:232.
32. Mansour R, Aboulghar M, Serour G. Dummy embryo transfer: a technique that minimizes the problems of embryo transfer and improves the pregnancy rate in human in vitro fertilization. Fertil Steril 1990; 54:578.
33. Prapas N, Prapas Y, Panagiotidis Y, et al. Cervical dilatation has a positive impact on the outcome of IVF in randomly assigned cases having two previous difficult embryo transfers. Hum Reprod 2004; 19:1791.
34. Noyes N, Licciardi F, Grifo J, et al. In vitro fertilization outcome relative to embryo transfer difficulty: a novel approach to the forbidding cervix. Fertil Steril 1999; 72:261.
35. Perez-Medina T, Bajo-Arenas J, Salazar F, et al. Endometrial polyps and their implication in the pregnancy rates of patients undergoing intrauterine insemination: a prospective, randomized study. Hum Reprod 2005; 20:1632.

36. Camus E, Poncelet C, Goffinet F, et al. Pregnancy rates after in vitro fertilization in cases of tubal infertility with and without hydrosalpinx: a meta-analysis of published comparative studies. Hum Reprod 1999; 14:1243.
37. Shelton KE, Butler L, Toner JP, et al. Salpingectomy improves the pregnancy rate in in vitro fertilization patients with hydrosalpinx. Hum Reprod 1996; 11:523.
38. Meyer WR, Castelbaum AJ, Somkuti S, et al. Hydrosalpinges adversely affect markers of endometrial receptivity. Hum Reprod 1997; 12:1393.
39. Surrey ES, Schoolcraft WB. Laparoscopic management of hydrosalpinges before in vitro fertilization–embryo transfer: salpingectomy versus proximal tubal occlusion. Fertil Steril 2001; 75:612.
40. McComb PF, Taylor RC. Pregnancy outcome after unilateral salpingostomy with a contralateral patient oviduct. Fertil Steril 2001; 76:1278.
41. de Wit W, Gowrising CJ, Kuik DJ, et al. Only hydrosalpinges visible on ultrasound are associated with reduced implantation and pregnancy rates after in vitro fertilization. Hum Reprod 1998; 13:1696.
42. Hill GA, Herbert CM, Fleischer AC, et al. Enlargement of hydrosalpinges during ovarian stimulation protocols for in vitro fertilization and embryo replacement. Fertil Steril 1986; 45:833.
43. Barnhart K, Dunsmoor-Su R, Coutifaris C. Effect of endometriosis on in vitro fertilization. Fertil Steril 2002; 77:1148.
44. Surrey ES, Silverberg KM, Surrey MW, et al. Effect of prolonged gonadotropin-releasing hormone agonist therapy on the outcome of in vitro fertilization–embryo transfer in patients with endometriosis. Fertil Steril 2002; 78:699.
45. Rickes D, Nickel I, Kropf S, et al. Increased pregnancy rates after ultralong postoperative therapy with gonadotropin-releasing hormone analogs in patients with endometriosis. Fertil Steril 2002; 78:757.
46. Karande VC, Lester RG, Muasher SJ, et al. Are implantation and pregnancy outcome impaired in diethylstilbestrol-exposed women after in vitro fertilization and embryo transfer. Fertil Steril 1990; 54:287.
47. Eldar-Geva T, Meagher S, Healy DL, et al. Effect of intramural, subserosal and submucosal uterine fibroids on the outcome of assisted reproductive technology treatment. Fertil Steril 1998; 70:687.
48. Hart R, Khalaf Y, Yeong C-T, et al. A prospective controlled study of the effect of intramural uterine fibroids on the outcome of assisted conception. Hum Reprod 2001; 16:2411–2417.
49. Stovall DW, Parrish SB, Van Voorhis BJ, et al. Uterine myomas reduce the efficacy of assisted reproduction cycles: results of a matched follow-up study. Hum Reprod 1998; 13:192.
50. Ramzy AM, Saltar M, Amin Y, et al. Uterine myomata and outcome of assisted reproduction. Hum Reprod 1998; 13:198.
51. Jun SH, Ginsberg ES, Racowsky C, et al. Uterine leiomyomas and their effect on in vitro fertilization outcome: a retrospective study. J Assist Reprod Genet 2001; 18:139.
52. Surrey ES, Lietz AK, Schoolcraft WB. Impact of intramural leiomyomata in patients with a normal endometrial cavity on in vitro fertilization–embryo transfer outcome. Fertil Steril 2001; 75:405.

53. Morshedi M, Oehninger S, Veeck LL, et al. Cryopreserved/thawed semen for in vitro fertilization: results from fertile donors and infertile patients. Fertil Steril 1990; 54:1093.
54. Hughes EG, Yeo J, Claman P, et al. Cigarette smoking and the outcomes of in vitro fertilization: measurement of effect size and levels of action. Fertil Steril 1994; 62:807.
55. Klonoff-Cohen H, Bleha J, Lam-Kruglick P. A prospective study of the effects of female and male caffeine consumption on the reproductive endpoints of IVF and gamete intrafallopian transfer. Hum Reprod 2002; 17:1746.
56. Fedorcsak P, Dale PO, Storeng R, et al. Impact of overweight and underweight on assisted reproduction treatment. Hum Reprod 2004; 19:2523.
57. Hakim RB, Gray RH, Zacur H. Alcohol and caffeine consumption and decreased fertility. Fertil Steril 1998; 70:632.
58. Juhl M, Olsen J, Andersen A-MN, et al. Intake of wine, beer and spirits and waiting time to pregnancy. Hum Reprod 2003; 18:1967.
59. Sanders KA, Bruce NW. Psychosocial stress and treatment outcome following assistant reproductive technology. Hum Reprod 1999; 14:1656.
60. Klonoff-Cohen H, Chu E, Natarajan L, et al. A prospective study of stress among women undergoing in vitro fertilization or gamete intrafallopian transfer. Fertil Steril 2001; 76:675.
61. Smeenk JMJ, Verhaak CM, Eugster A, et al. The effect of anxiety and depression on the outcome of in vitro fertilization. Hum Reprod 2001; 16:1420.
62. Domar AD, Clapp D, Slawsby EA, et al. Impact of group psychological interventions on pregnancy rates in infertile women. Fertil Steril 2000; 73:805.

3

Testing Ovarian Reserve

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INTRODUCTION

Both the quantity of eggs and their quality are strong influences on IVF outcome. Markers of ovarian reserve, such as basal follicle-stimulating hormone (FSH) and basal antral follicle (BAF) counts, are good predictors of the quantity of eggs which can be induced to grow. However, the quality of those eggs seems better predicted by the age of the women. In women past age 40, current success rates are low overall, even among those who make many eggs; at this age, quantity does not make up for quality. By contrast, young women with limited ovarian reserve can have good success rates despite their limited cohort of eggs, because the eggs themselves are of high quality; here quality matters more than quantity. The ramifications of these observations include the following: Diminished ovarian reserve should not be used as an exclusionary criterion in young women, because overall they still have satisfactory pregnancy rates, though their risk of cancellation is increased. In women past age 40, normal ovarian reserve testing is not reassuring because even reduced egg quality is likely to limit the opportunity for successful pregnancy no matter how many eggs are available.

Seventeen years ago, Muasher and Oehninger from the Norfolk in vitro fertilization (IVF) program reported that basal follicle-stimulating hormone (FSH) levels predicted ovarian response and pregnancy outcome in IVF cycles (1). Since then, more than a hundred articles have sought to refine our understanding of the link between markers of "ovarian reserve"

and pregnancy in assisted reproductive technology (ART). Although, there have been important refinements to our original understanding, the essence of the initial message has only been confirmed in the numerous studies that followed. In this chapter, we will consider the biological basis for the links among the markers of ovarian reserve, the reserve itself, and the pregnancy potential. We will also review the original and newer elements of this understanding.

PHYSIOLOGY OF OVARIAN RESERVE

Ovaries contain all the eggs they will ever have before birth. Depletion of this supply begins before birth, and continues until menopause, when the endowment is gone (Fig. 1). The rate of this depletion is fairly constant over a woman's life span, but accelerates at around 37 years of age on average. At the beginning of every menstrual cycle, a fixed proportion of all remaining eggs acquires gonadotropin sensitivity. In natural cycles, all but one of these recruitable eggs undergo atresia, but the size of the recruitable cohort correlates with the woman's age. Given that the overall number of eggs in younger women is higher than in later years, the size of the cohort of recruitable eggs in younger women is much larger.

As the number of remaining eggs decrease with age, certain predictable concomitants have been observed. These include physical manifestations, such as smaller ovaries and fewer antral follicles, but also hormonal events, such as elevations of basal FSH and shorter follicular phases (2,3). Morris et al. (4) have confirmed that the number of visible antral follicles on ultrasound correlates with the actual number in the primordial follicle pool.

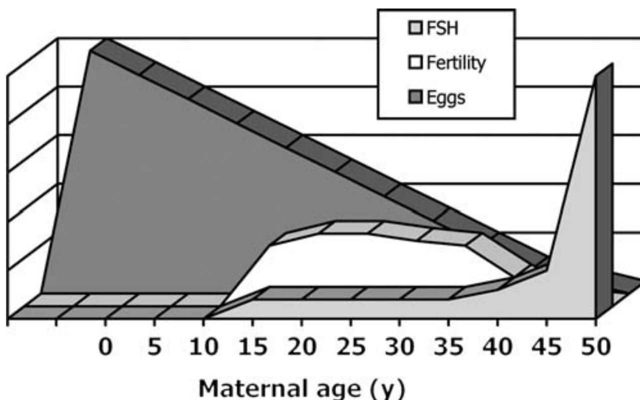

Figure 1 At birth, a women has all the eggs she will ever have, and steadily looses them thereafter, until none remain at menopause. As her age increases and the supply diminishes, fertility declines. This fall in fertility is often signaled by a rise in basal FSH levels. *Abbreviation*: FSH, follicle-stimulating hormone.

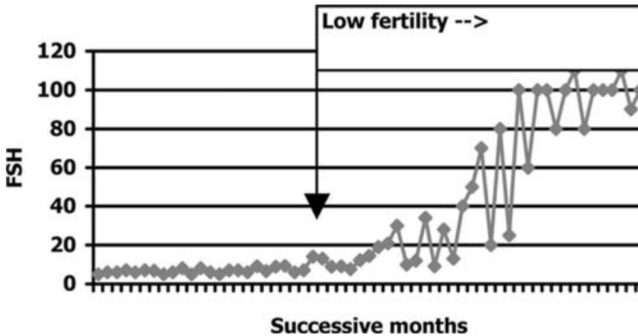

Figure 2 The basal FSH level at the beginning of menstrual cycles can be divided into three phases. In the first phase, fertility is normal and FSH is always low. In the second phase, FSH is intermittently elevated and fertility is declining. In the last phase, FSH is always elevated and fertility is nil. *Abbreviation*: FSH, follicle-stimulating hormone.

One clinically useful indicator of follicle depletion is elevated FSH. Although basal (i.e., days 2–5 of the menstrual cycle) FSH fluctuates somewhat from cycle to cycle, three phases are distinguished: (i) Up until the time that egg supply begins to become limited, basal FSH is never elevated. (ii) Once menopause is well established, basal FSH is always elevated and (iii) During the intermediate stage, FSH is sometimes elevated and sometimes normal (Fig. 2). During this intermediate phase, however, fecundity is reduced whether or not the FSH is elevated during a particular cycle. Several studies have demonstrated that the ovarian response and the pregnancy rate in cycles with normal FSH is low if any prior cycle displayed an abnormal FSH.

ORIGINAL UNDERSTANDINGS

Many elements of the initial reports are still valid, including:

1. High age is limiting even with normal FSH. Original reports demonstrated lower pregnancy rates in women past 40 years of age, no matter their basal FSH level (5). Even with today's IVF, deliveries in women past age 42 years are uncommon, and past 45 years are rare.
2. High FSH is limiting even with normal age. The original reports saw a declining pregnancy rate as FSH rose above 20 IU/L and no ongoing pregnancies beyond an FSH of 25 IU/L (5,6). Although the assay has since changed and altered these cutoffs, there is still a threshold above which declining performance (egg production and pregnancy rate) is detected, and a higher threshold above which egg production is quite limited, and almost no pregnancies have occurred. Although this higher cutoff is not

commonly reached [only 5% of cases in one study (7)], it reliably predicts low delivery rates.

3. Cutoffs for FSH depend on the lab test employed. Up through the early 1990s, the most commercial assays reported FSH levels about twice as high as those now in wide use. However, the typical assay method switched from a standard radioimmunoassay to a double antibody approach, and the assay standard changed. Consequently, the normal FSH cutoff of 20 IU/L is now just 10 IU/L.
4. The highest FSH is the best predictor of ovarian reserve. Several early reports demonstrated the futility of delaying treatments until a cycle with a normal FSH occurs. More recent studies have continued to affirm this effect (7–9). Once an FSH elevation is observed, egg production capacity will be limited thereafter. This is to be expected, given the on-again, off-again nature of basal FSH elevations once egg numbers become critically short (Fig. 2).
5. Prediction of ovarian reserve is easier than predicting pregnancy. Basal FSH levels are better able to predict outcomes more closely related to ovarian function, such as cancellation ($R^2 = 77\%$), follicles aspirated ($R^2 = 35\%$), and oocytes retrieved ($R^2 = 21\%$) than more distal events such as pregnancy rate ($R^2 = 4\%$) (5,10,11). In this regard, FSH is a better guide to selecting stimulation strength than who will become pregnant with treatment.

REFINED UNDERSTANDINGS

1. *High age and high FSH affect delivery rates, but in different ways.* FSH is the better predictor of the number of eggs that can be induced to grow by gonadotropin administration and, consequently, cancellation rate (12–16). Age, on the other hand, is the better predictor of embryo implantation and miscarriage rate (13,16–18). As prospects for delivery are affected by both quantitative and qualitative deficiencies in eggs, both age and FSH are important (Fig. 3).

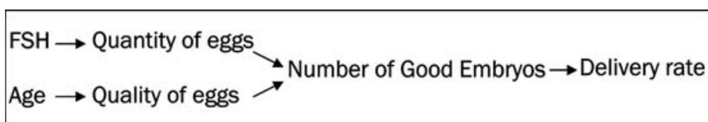

Figure 3 The chance for successful pregnancy outcome in in vitro fertilization (IVF) is influenced by both quantitative and qualitative factors regarding eggs. The quantitative aspect seems best predicted by various markers of ovarian reserve such as basal FSH. The qualitative aspect seems best predicted by maternal age, and is manifest in implantation rates. *Abbreviation*: FSH, follicle-stimulating hormone.

Initial claims (by this author) that FSH was more predictive of outcome than age reflected a lack of understanding that both ovarian reserve and age are important independent predictors. The apparent strength of one over the other in any particular study has more to do with the ranges of FSH levels and age in a particular study than in any underlying physiological principle. Furthermore, at extremes of either age or FSH (e.g., age > 45 years, FSH > 20 IU/L), fertility is essentially nil (7,11,19,20). For instance, in one large study, high FSH levels were associated with a very low pregnancy rate (2.7%) even among women under age 35 years (21). And women aged 43 years or more have delivery rates under 3% per attempt in every annual report of U.S. ART outcomes. Clearly both factors are important predictors.

2. *Young women with moderate elevations of FSH will make fewer eggs and have a higher risk of cycle cancellation, but if eggs are retrieved, they have reasonable chances for pregnancy*. This "protective" effect of young age was not seen in the original study (5), but has been seen repeatedly since (22–26) as described below.

   For example, the van Rooij et al. study (23) noted that in women under age 40 years with elevated FSH, the risk for cancellation was high, but the pregnancy rate among those proceeding to transfer was good (Table 1).

   In another informative study (27), mild elevations of FSH predicted the need for more stimulation to get an acceptable ovarian response. Even with this adjustment, a lower response was in evidence, but enough eggs were produced to achieve a roughly equivalent transfer and pregnancy rate (Table 2). However, as FSH became markedly elevated, pregnancy rates fell as stimulation adjustments were unable to compensate for diminished ovarian responsiveness.

Table 1 Comparison of Outcomes in Younger Patients with Diminished Ovarian Reserve to Older Patients with Normal Ovarian Reserve

	<40 years with FSH 15+	41+ years with FSH <15
N	36	50
% canceled	31	8
% embryos implanted	34	11
% clinically pregnant	40	13
% ongoing pregnant	25	10

Abbreviation: FSH, follicle-stimulating hormone.
Source: From Ref. 23.

Table 2 Influence of Basal Follicle-Stimulating Hormone on Ovarian Response and Pregnancy Outcomes

	Basal FSH (IU/L)			
	<10	10–15	15–20	20+
No. of amps	27.6	38.2		
Estradiol at hCG	2391	1277		
No. of eggs retrieved	13.1	6.5		
No. of good embryos	2.9	2.5	2.1	1.7
Clinical pregnancy rate (%)	24.6	23.4	13.6	5.7
Live birth rate (%)	19.6	18.2	13.6	2.9

Abbreviations: FSH, follicle-stimulating hormone; hCG, human chorionic gonadotrophin.

The strength of the relation between basal hormone markers and ovarian reserve is enhanced with luteal estradiol administration (28).

The combined effect of age and FSH on ultimate delivery rate is illustrated in Figure 4. Note that women beyond age 42 years are unlikely to deliver no matter what their basal FSH might be; this reflects the significant reduction in egg quality (i.e., implantation

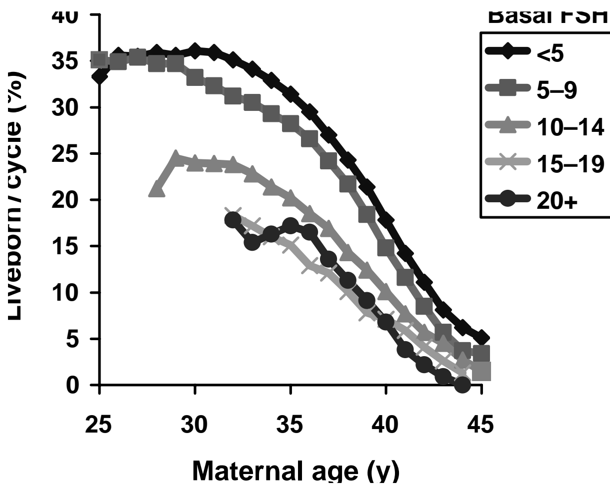

Figure 4 Simultaneous consideration of age and FSH is important for understanding the chance for successful pregnancy in vitro fertilization (IVF) (theoretical model). High age (> 42 years) is a significant impediment no matter what the ovarian reserve, but high FSH also compromises success. Note that in young women, the success rate exceeds that for older women even when FSH is elevated. This supports the observation that in young women, even a few eggs can be sufficient; these women can and should be given the chance to try IVF as long as they are counseled regarding the increased risk of cancellation. *Abbreviation*: FSH, follicle-stimulating hormone.

potential) which is nearly universally seen at this age independent of predicted or actual ovarian responsiveness. Also note that although younger women's success is dependent on basal FSH, even those with FSH elevations have a chance for pregnancy.

3. *Other Markers of Ovarian Reserve*

   a. *An exaggerated FSH/luteinizing hormone (LH) ratio*, even with normal FSH, is a sign of diminished ovarian reserve. In fact, the ratio of FSH/LH appears to be a clinically useful index, suggesting a PCO-like high response when LH exceeds FSH on one end of the spectrum, to diminished ovarian reserve when FSH exceeds LH (29–32) at the other. It is interesting to note that some PCO will develop regular cycles as their egg supply declines (33), as their FSH levels rise and their inhibin B levels fall.
   b. *Decreased early follicular phase inhibin B* levels may occur before increases in FSH are observed (34).
   c. *Antral follicle count* is a good predictor of ovarian reserve (35–38), and in some but not all studies appear better than the usual endocrine markers (including FSH) (35–39). Cancellation rate and egg production are better predicted by the antral follicle count than pregnancy rate. Ovarian volume seems less predictive than the follicle count (40).
   d. *Increased day 3 estradiol* has been associated with both diminished ovarian reserve and enhanced ovarian reserve (ala PCO). This makes interpretation of this test problematic without further information. Those with diminished ovarian reserve display a high estradiol because of hurried folliculogenesis and will have a low antral follicle count. Those with PCO can display a high estradiol as their many antral follicles each make a bit of estradiol. Interestingly, cancellation is increased with either low (<20 pg/mL) or high (>80 pg/mL) estradiol levels (41,42), but these levels did not predict pregnancy rate in those not canceled. The combined FSH and estradiol in screening for diminished ovarian reserve appears to be more sensitive than either test alone (43).
   e. *Provocative tests of ovarian reserve*, such as exogenous FSH ovarian reserve test (44), GnRH agonist stimulation test (45), and Clomiphene citrate challenge test (CCCT) (46) are more sensitive indicators of ovarian reserve than basal tests. Among these, the CCCT seems to best predict ovarian reserve (47,48). A large meta-analysis (49) recently showed both FSH and the CCCT to be highly specific (each >98%, i.e., an abnormal test result predicting no pregnancy); however, the CCCT was more sensitive (detecting about 25% of those not pregnant vs. only 6% for FSH alone). In the CCCT,

an abnormal day 10 appears to carry the same poor prognosis as does an abnormal day 3; the prognosis is even worse if both are abnormal (50).

f. *Premature luteinization and a short follicular phase* can be signs of diminished ovarian reserve (38,51,52).

4. *Miscarriage risk* is increased in those with diminished ovarian reserve (53–55).
5. *Birth defect risks* may be increased in those with diminished ovarian reserve (56).
6. *Ovarian Reserve Effects in Natural Cycles*. In a small study ($n = 129$) of a general population of couples trying to conceive, FSH was not predictive of pregnancy or miscarriage (57). Even in general subfertility population, one small study of women with an FSH > 10 IU/L did not predict more time to pregnancy, fewer pregnancies, or fewer deliveries over a year's effort to conceive naturally (58). In another similar study of 103 young couples (average age of 33.2 years), pregnancy in the first year of unassisted reproduction was influenced by the woman's age, but not basal FSH, estradiol, or the basal follicle count (59). Insofar as natural cycles generally produce and release only one egg and that markers of ovarian reserve are more predictive of egg production capacity than egg quality, this absence of effect in unstimulated cycles is not surprising.
7. *When an Elevated FSH does not Signal Trouble*. On occasion, an elevated FSH may not signal quantitative limitations in egg production capacity per se, such as in cases of familial twinning or in the presence of heterophilic antibodies to FSH. Further, although in many cases a rise in FSH signals both quantitative and qualitative reductions in eggs, after ovarian tissue loss (surgical extirpation for cysts, endometriomas, etc.), one would expect only a quantitative reduction.

CONTROVERSIES

Is BAF a Sufficient Test?/Can We Abandon Day 3 Bloods?

For predicting the number of eggs retrieved, basal antral follicle count (BAF) may be the best single test, but endocrine markers (FSH and inhibin) add additional predictive power to BAF alone (34). In this study, egg number was not predicted by age, basal E2, and total ovarian volume.

Since the Predictive Power for Delivery is Low, does any Testing Matter?

IVF is an expensive and inconvenient remedy for infertility. Basal markers such as FSH and BAF are good guides for ovarian response potential and

are an important tool for picking a stimulation protocol of appropriate strength. And although our ability to predict pregnancy is generally poor with these same markers, there remains a small group with high FSH levels whose chance for pregnancy is almost nil (11). For instance, in the original study of 1478 cycles, those with a basal FSH over 25 IU/L (5% of the group) had a 5.5-fold increase in poor response and a 12-fold increase in nonpregnancy outcome (5). Such effects are not unimportant, even if they identify a small subset of the entire IVF population.

SUMMARY

Various means of predicting ovarian response have been identified. Of these, basal FSH and ultrasound estimates of antral follicle count are the most useful. Adjusting stimulation strength to the expected ovarian reserve is a useful clinical practice. However, ovarian reserve is a relatively weak predictor of pregnancy potential, except when it is extremely low (which is uncommon overall). In the bulk of cases, maternal age is an important influence on pregnancy potential that operates independent of ovarian reserve (although the two are in many cases confounded).

As a rule of thumb, FSH and BAF are better predictors of ovarian reserve than is the age of the women, whereas age is a better predictor of the implantation potential of resulting embryos.

The ramifications of these observations include the following: abnormal tests of ovarian reserve are the best predictors we have of egg production capacity, but are not perfect. Thus diminished ovarian reserve should not be used as an exclusionary criterion in young women because overall they still have satisfactory pregnancy rates, although their risk of cancellation is increased. In women past age 40 years, normal ovarian reserve testing is not particularly reassuring because reduced egg quality is likely to limit the opportunity for successful pregnancy even when many eggs are available.

REFERENCES

1. Muasher SJ, Oehninger S. The value of basal and/or stimulated serum gonadotropin levels in prediction of stimulation response and in vitro fertilization outcome. Fertil Steril 1988; 50(2):298–307.
2. Scheffer GJ, Broekmans FJM, et al. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. Fertil Steril 1999; 72(5):845–851.
3. Erdem A, Erdem M, Biberoglu K, Hayit O, Arslan M, Gursoy R. Age-related changes in ovarian volume, antral follicle counts and basal FSH in women with normal reproductive health. J Reprod Med 2002; 47(10):835–839.
4. Morris JL, Thyer AC, Soules MR, Klein NA. Antral follicle count by transvaginal ultrasound is reflective of the actual primordial follicle pool. Fertil Steril 2002; 78(1001):S3–S4.

5. Toner JP, Philput CB, Jones GS, Muasher SJ. Basal follicle-stimulating hormone level is a better predictor of in vitro fertilization performance than age. Fertil Steril 1991; 55(4):784–791.
6. Scott RT, Toner JP, Muasher SJ, Oehninger S, Robinson S, Rosenwaks Z. Follicle-stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome. Fertil Steril 1989; 51(4):651–654.
7. Bancsi LF, Huijs AM, den Ouden CT, et al. Basal follicle-stimulating hormone levels are of limited value in predicting ongoing pregnancy rates after in vitro fertilization. Fertil Steril 2000; 73(3):552–557.
8. Pal L, Witt BR. Maximal historical FSH is a better predictor of cycle outcome than the baseline FSH for the IVF cycle. Fertil Steril 2002; 78(1001):S135.
9. Lass A, Gerrard A, Abusheikha N, Akagbosu F, Brinsden P. IVF performance of women who have fluctuating early follicular FSH levels. J Assist Reprod Genet 2000; 17(10):566–573.
10. Tinkanen H, Blauer M, Laippala P, Tuohimaa P, Kujansuu E. Prognostic factors in controlled ovarian hyperstimulation. Fertil Steril 1999; 72(5):932–936.
11. Bancsi LF, Broekmans FJ, Mol BW, Habbema JD, te Velde ER. Performance of basal follicle-stimulating hormone in the prediction of poor ovarian response and failure to become pregnant after in vitro fertilization: a meta-analysis. Fertil Steril 2003; 79(5):1091–1100.
12. Ghazeeri GS, Chuang A, Barker LJ, Ke RW, Kutteh WH. Elevated day 10 FSH is predictive of pregnancy loss in patients undergoing IVF. Fertil Steril 2001; 76(3 suppl 1):S209.
13. Akande VA, Fleming CF, Hunt LP, Keay SD, Jenkins JM. Biological versus chronological ageing of oocytes, distinguishable by raised FSH levels in relation to the success of IVF treatment. Hum Reprod 2002; 17(8):2003–2008.
14. Magarelli PC, Pearlstone AC, Buyalos RP. Discrimination between chronological and ovarian age in infertile women aged 35 years and older: predicting pregnancy using basal follicle stimulating hormone, age and number of ovulation induction/intra-uterine insemination cycles. Hum Reprod 1996; 11(6):1214–1219.
15. Poe-Zeigler R, Toner JP, Oehninger S, Muasher SJ. Basal FSH affects IVF pregnancy rates primarily by influencing oocyte numbers and not their quality. Abstract #O-014, Program Supplement. American Fertility Society Annual Meeting, San Antonio, Texas, November 5–10, 1994:S8 (oral presentation).
16. Sharif K, Elgendy M, Lashen H, Afnan M. Age and basal follicle stimulating hormone as predictors of in vitro fertilization outcome. Br J Obstet Gynecol 1998; 105(1):107–112.
17. Chuang CC, Chen CD, Chao K-H, Chen S-U, Ho H-N, Yang Y-S. Age is a better predictor of pregnancy potential than basal follicle-stimulating hormone levels in women undergoing in vitro fertilization. Fertil Steril 2003; 79(1):63–68.
18. Hull MG, Fleming CF, Hughes AO, McDermott A. The age-related decline in female fecundity: a quantitative controlled study of implanting capacity and survival of individual embryos after in vitro fertilization. Fertil Steril 1996; 65(4):783–790.
19. Lass A, Croucher C, Duffy S, Dawson K, Margara R, Winston RML. One thousand initiated cycles of in vitro fertilization in women $\geq$40 years of age. Fertil Steril 1998; 70(6):1030–1034.

20. Tummon IS, Hardy I, Lee M, Cardone VR, Seibel M, Summers M. Lifetime high day 2 FSH and diagnostic accuracy in IVF. Fertil Steril 2001; 76(3 suppl 1): S232–S233.
21. Levi AJ, Raynault MF, Bergh PA, Drews MR, Miller BT, Scott RT Jr. Reproductive outcome in patients with diminished ovarian reserve. Fertil Steril 2001; 76(4):666–669.
22. Pal L, Cohen H, Witt B. Pregnancy rates with IVF remain significant despite elevated maximal basal FSH levels in younger women. Fertil Steril 2002; 78(1001):S245.
23. van Rooij IAJ, Bancsi LFJMM, Broekmans FJM, Looman CWN, Habbema JDF, te Velde ER. Women older than 40 years of age and those with elevated follicle-stimulating hormone levels differ in poor response rate and embryo quality in in vitro fertilization. Fertil Steril 2003; 79(3):482–488.
24. Hanoch J, Lavy Y, Holzer H, et al. Young low responders protected from the untoward effects of reduced ovarian response. Fertil Steril 1998; 69(6): 1001–1004.
25. Ulug U, Ben-Shlomo I, Turan E, Erden HF, Akman MA, Bahceci M. Conception rates following assisted reproduction in poor responder patients: a retrospective study in 300 consecutive cycles. Reprod Biomed Online 2003; 6(4):439–443.
26. Abdalla H, Thum MY. An elevated basal FSH reflects a quantitative rather than qualitative decline of the ovarian reserve. Hum Reprod 2004; 19:893–898.
27. Hur K, Han K, Byun H, Yeum H, Choi S, Jun J. Mildly elevated basal FSH does not predict poor outcome of assisted reproductive technologies. Fertil Steril 2002; 78(1001):S249.
28. Fanchin R, Cunha-Filho J, Schonauer LM, Righini C, de Ziegler D, Frydman R. Luteal estradiol administration strengthens the relationship between day 3 follicle-stimulating hormone and inhibin B levels and ovarian follicular status. Fertil Steril 2003; 79(3):585–589.
29. Kligman I, Rosenwaks Z. Differentiating clinical profiles: predicting good responders, poor responders, and hyperresponders. Fertil Steril 2001; 76(6): 1185–1190.
30. Barroso G, Oehninger S, Monzo A, Kolm P, Gibbons WE, Muasher SJ. High LH:FSH ratio and low LH levels in basal cycle day 3: impact on follicular development and IVF outcome. J Assist Reprod Genet 2001; 18(9):499–505.
31. Noci I, Maggi M, Fuzzi B, Biagiotti R, Ricci F, Marchionni M. Effects of low day 3 luteinizing hormone levels on in vitro fertilization treatment outcome. Gynecol Endocrinol 2000; 14(5):321–326.
32. Ho JYP, Guu HF, Yi YC, Chen MJ, Ho ESC. The serum follicle-stimulating hormone-to-luteinizing hormone ratio at the start of stimulation with gonadotropins after pituitary down-regulation is inversely correlated with a mature oocyte yield and can predict "low responders." Fertil Steril 2005; 83:883–888.
33. Elting MW, Kwee J, Korsen TJ, Rekers-Mombarg LT, Schoemaker J. Aging women with polycystic ovary syndrome who achieve regular menstrual cycles have a smaller follicle cohort than those who continue to have irregular cycles. Fertil Steril 2003; 79(5):1154–1160.

34. Seifer DB, Scott RT Jr., Bergh PA, et al. Women with declining ovarian reserve may demonstrate a decrease in day 3 serum inhibin B before a rise in day 3 follicle-stimulating hormone. Fertil Steril 1999; 72(1):63–65.
35. Bancsi LF, Broekmans FJM, et al. Predictors of poor ovarian reserve in in vitro fertilization: a prospective study comparing basal markers of ovarian reserve. Fertil Steril 2002; 77(2):328–336.
36. Pellicer A, Ardiles G, Neuspiller F, Remohi J, Simon C, Bonilla-Musoles F. Evaluation of the ovarian reserve in young low responders with normal basal levels of follicle-stimulating hormone using three-dimensional ultrasonography. Fertil Steril 1998; 70(4):671–675.
37. Frattarelli JL, Lauria-Costab DF, Miller BT, Bergh PA, Scott RT Jr. Basal antral follicle number and mean ovarian diameter predict cycle cancellation and ovarian responsiveness in assisted reproductive technology cycles. Fertil Steril 2000; 74(3):512–517.
38. Yong PYK, Baird DT, JooThong K, McNeilly AS, Anderson RA. Prospective analysis of the relationships between the ovarian follicle cohort and basal FSH concentration, the inhibin response to exogenous FSH and ovarian follicle number at different stages of the normal menstrual cycle and after pituitary down-regulation. Hum Reprod 2003; 18:35–44.
39. Broekmans FJ, Faddy M, te Velde ER. Ovarian reserve and reproductive age may be determined from measurement of ovarian volume by transvaginal sonography. Hum Reprod 2005; 20:1114–1115.
40. Beckers NG, Macklon NS, Eijkemans MJ, Fauser BC. Women with regular menstrual cycles and a poor response to ovarian hyperstimulation for in vitro fertilization exhibit follicular phase characteristics suggestive of ovarian aging. Fertil Steril 2002; 78(2):291–297.
41. Phelps JY, Levine AS, Hickman TN, Zacur HA, Wallach EE, Hinton EL. Day 4 estradiol levels predict pregnancy success in women undergoing controlled ovarian hyperstimulation for IVF. Fertil Steril 1998; 69(6):1015–1019.
42. Frattarelli JL, Bergh PA, Drews MR, Sharara FI, Scott RT Jr. Evaluation of basal estradiol levels in assisted reproductive technology cycles. Fertil Steril 2000; 74(3):518–524.
43. Ranieri DM, Quinn F, Makhlouf A, Khadum I, Ghutmi W, McGarrigle H, et al. Simultaneous evaluation of basal follicle-stimulating hormone and 17β-estradiol response to gonadotropin-releasing hormone analogue stimulation: an improved predictor of ovarian reserve. Fertil Steril 1998; 70(2):227–233.
44. Fanchin R, de Ziegler D, Olivennes F, Taieb J, Dzik A, Frydman R. Exogenous follicle stimulating hormone ovarian reserve test (EFORT): a simple and reliable screening test for detecting 'poor responders' in in vitro fertilization. Hum Reprod 1994; 9(9):1607–1611.
45. Winslow KL, Toner JP, Brzyski RG, Oehninger SC, Acosta AA, Muasher SJ. The gonadotropin-releasing hormone agonist stimulation test a sensitive predictor of performance in the flare-up in vitro fertilization cycle. Fertil Steril 1991; 56(4):711–717.
46. Navot D, Rosenwaks Z, Margalioth EJ. Prognostic assessment of female fecundity. Lancet 1987; 2(8560):645–647.

47. Gulekli B, Bulbul Y, Onvural A, et al. Accuracy of ovarian reserve tests. Hum Reprod 1999; 14(1):2822–2826.
48. Bukman A, Heineman MJ. Ovarian reserve testing and the use of prognostic models in patients with subfertility. Hum Reprod Update 2001; 7(6):581–590.
49. Jain T, Soules M, Collins JA. Comparison of basal follicle-stimulating hormone versus the clomiphene citrate challenge test for ovarian reserve screening. Fertil Steril 2004; 82:180–185.
50. Yanushpolsky EH, Hurwitz S, Tikh E, Racowsky C. Predictive usefulness of cycle day 10 follicle-stimulating hormone level in a clomiphene citrate challenge test for in vitro fertilization outcome in women younger than 40 years of age. Fertil Steril 2003; 80(1):111–115.
51. Younis JS, Matilsky M, Radin O, Ben-Ami M. Increased progesterone/estradiol ratio in the late follicular phase could be related to low ovarian reserve in in vitro fertilization–embryo transfer cycles with a long gonadotropin-releasing hormone agonist. Fertil Steril 2001; 76(2):294–299.
52. Younis JS, Haddad S, Matilsky M, Ben-Ami M. Premature luteinization: could it be an early manifestation of low ovarian reserve. Fertil Steril 1998; 69(3):461–465.
53. Hofmann GE, Khoury J, Thie J. Recurrent pregnancy loss and diminished ovarian reserve. Fertil Steril 2000; 74(6):1192–1195.
54. Trout SW, Seifer DB. Do women with unexplained recurrent pregnancy loss have higher day 3 serum FSH and estradiol values? Fertil steril 2000; 74(2): 335–337.
55. Nasseri A, Mukherjee T, Grifo JA, Noyes N, Krey L, Cooperman AB. Elevated day 3 serum follicle-stimulating hormone and/or estradiol may predict fetal aneuploidy. Fertil Steril 1999; 71(4):715–718.
56. van Montfrans J, van Hooff M, Martens F, van Vugt JMG, Lambalk N. Basal FSH concentrations as marker of ovarian aging are not related to pregnancy outcome in a general population of women over 30 years. Fertil Steril 2002; 78(1001):S223–S224.
57. van Montfrans JM, Hoek A, van Hooff MHA, de Koning Ch, Tonch N, Lambalk CB. Predictive value of basal follicle-stimulating hormone concentrations in a general subfertility population. Fertil Steril 2000; 74(1):97–103.
58. Broekmans F, van Rooy I, te Velde E. The predictive role of ovarian reserve tests in the normal population. Fertil Steril 2002; 78(1001):S217.
59. Lambalk CB. Value of elevated basal follicle-stimulating hormone levels and the differential diagnosis during the diagnostic subfertility work-up. Fertil Steril 2003; 79(3):489–490.

4

Basics of Ovarian Stimulation

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INTRODUCTION

In this chapter, we shall review human ovarian physiology, clinical reproductive endocrinology, and their application for ovarian stimulation for in vitro fertilization (IVF). The chapter will focus on a practical clinical approach as used daily in a large IVF program. Clinical guidelines, as suggested by the National Collaborating Centre for Women's and Children's Health, commissioned by the U.K. National Institute for Clinical Excellence (NICE), will be used throughout this chapter to highlight the evidence base for the basics of ovarian stimulation. Reference to other skills in clinical IVF ovarian stimulation will be directed to other chapters in this book. For example, the reader is referred to Chapters 2 and 3 (patient pre-treatment: testing ovarian reserve) for a practical approach to endocrine evaluation before IVF treatment.

The use of gonadotrophin-releasing hormone (GnRH) antagonists in human IVF is discussed in Chapter 5. The use of the natural cycle for in vitro oocyte maturation and subsequent IVF is outside the scope of this chapter and is covered in Chapter 8.

HUMAN OVARIAN PHYSIOLOGY

The initiation of follicular growth is a continuous process that is believed to not depend on hormonal stimulation. About 50 follicles each day begin to grow in the human ovaries. Most follicles undergo rapid atresia. This total growth phase occurs over approximately 90 days or over three ovarian cycles. However, under the hormonal changes in the late luteal phase and the beginning of the new cycle, a group of follicles will respond to small rises in serum follicle-stimulating hormone (FSH) by processing to the preantral stage (Fig. 1) (1). At this stage, the follicle is about 200 μm with multiple granulosa cell layers. Under the influence of FSH, the number of FSH receptors on the granulosa cell increases to about 1500 receptors per cell, and, at the same time, the granulosa cells start to produce estradiol 17β (E2) by aromatizing androgens that are supplied by the theca cells under luteinizing hormone (LH) stimulation. E2 and FSH together cause proliferation of granulosa cells and increase the number of FSH receptors on the granulosa cell plasma membrane. Production of follicular fluid increases. Its accumulation in the intracellular space eventually forms a cavity—the antrum. The size of the antral follicle is now about 500 μm in diameter (2,3).

By days 5–7, the dominant follicle is selected because of its ability to convert androgens to estrogens. The other follicles will not continue to grow and will undergo atresia. The dominant follicle continues to grow and secretes E2 and inhibin B that exert negative feedback on FSH production, causing a decline in serum FSH levels. FSH induces the appearance of LH receptors on granulosa cells, a process which is augmented by the concomitant presence of high estrogen levels. E2 production gradually increases, and plasma E2 levels achieve a threshold concentration required for the generation of the LH surge that starts 14–24 hr after the serum E2 reaches peak concentration.

Intra-ovarian peptides play important roles in modulating gonadotropin effects on ovarian function. At least 33 putative paracrine–autocrine regulators of follicular growth and atresia are identified (4). FSH enhances the secretion of most of them by granulosa cells. Insulin-like growth factor I (IGF-I) augments FSH-mediated aromatization, granulosa cell mitogenesis, and the induction of LH receptors. Inhibin, in addition to its endocrine negative effect on FSH secretion, inhibits aromatization and stimulates LH-induced androgen production by theca cells (5). Activin has a positive effect on aromatization (6,7), granulosa cell mitogenesis (8,9), and a negative paracrine action on LH-induced androgen production by theca cells (5). Activin is also involved in the regulation of apoptosis in the ovary (10). Follistatin, the third member in the inhibin/activin family, is antagonistic to activin (11). Vascular endothelial growth factor (VEGF) and growth factors such as epidermal growth factor (EGF) and transforming growth factors α (TGFα) also play important roles in modulating gonadotropin effects on ovarian function (12,13).

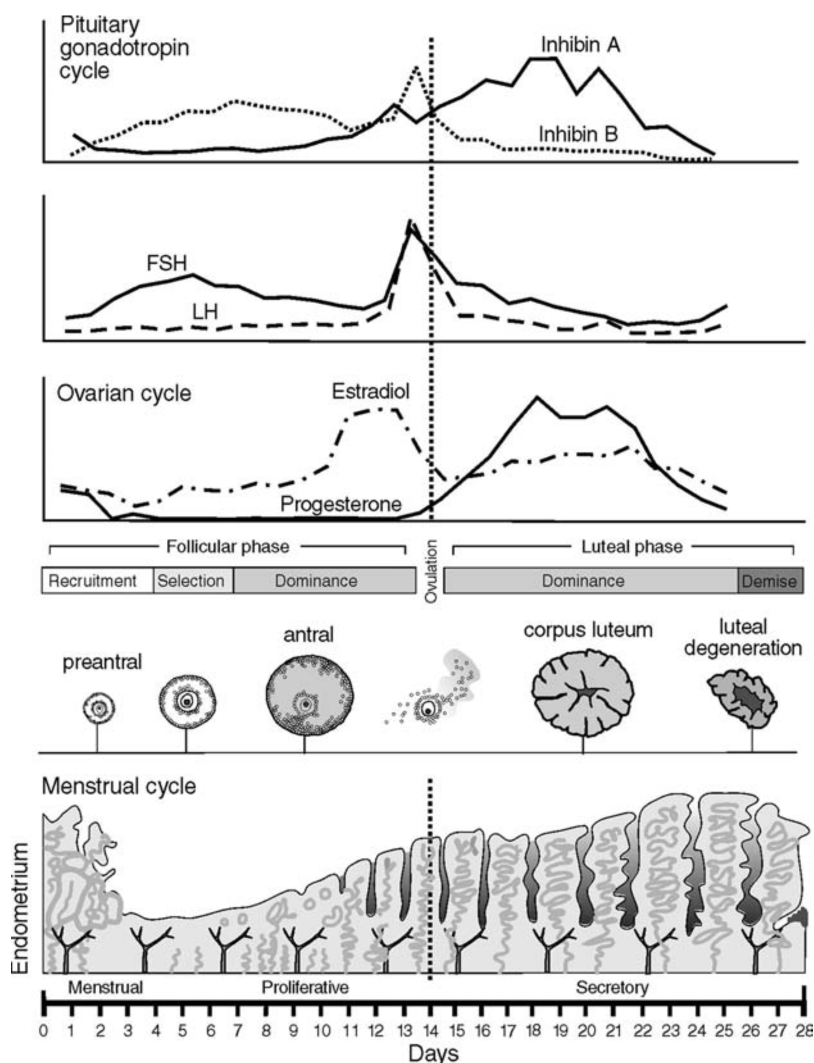

Figure 1 A schematic representation of the ovarian and menstrual cycles. *Abbreviations*: FSH, follicle-stimulating hormone; LH, luteinizing hormone.

The LH surge initiates luteinization and the beginning of progesterone production by the granulosa cells of the dominant follicle. It is also responsible for the resumption of meiosis in the oocyte (14). Activin promotes and inhibin inhibits the LH surge and superovolution in a rat model (15). LH stimulates the synthesis of cytokines, the best known of which is interleukin-1 (IL-1) which modulates activation of prostaglandins (16) and the proteolytic

cascade that are essential for follicular rupture (17). Ovulation occurs 24–36 hr after the onset of the LH surge, when the follicle, which is about 20 mm, ruptures and the oocyte is released from the ovary.

After ovulation, the dominant follicle becomes the corpus luteum (Fig. 1), producing progesterone, E2, and inhibin, which suppress the growth of new follicles in the ovary. At the end of the cycle, luteolysis causes decline in both steroids and inhibin, allowing the elevation in serum LH and FSH that is necessary for the initiation of a new cycle.

Intra-Ovarian Growth Factors

As introduced above, ovarian follicles produce a number of TGFβ-related proteins. Anti-mullerian hormone, TGFβs, activins, and inhibins are produced by granulosa cells. Both bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) are expressed exclusively by the oocyte of several species (18–20) BMP15 and GDF9 stimulate granulosa cell mitogenesis (21). BMP15 is a potent inhibitor of FSH-receptor expression and participates in negative feedback influencing granulosa cell mitosis (22). BMP6 is also expressed in the oocyte and inhibits FSH action, probably by downregulation of adenylate cyclase (23). There is a rapid decrease in BMP6 concentration in granulosa cells around the time of dominant follicle selection.

BMP Receptors

Members of the TGFβα superfamily signal through the activin/TGFβ and/or BMP pathways (24). The BMP receptors (BMPRIA/ALK3, BMPRIB/ALK6, and BMPR2) are transmembrane serine/threonine kinases closely related to the transforming growth factor beta receptors (TGFBRI/ALK5, TGFBR2) and activin receptors (ACVR1, ACVR1B, ACVR2, and ACVR2B). BMP receptors are expressed in granulosa cells and oocytes (25) and the BMPs exert their biological actions by forming heteromeric complexes with type I and II receptors (26). Ligands bind to the type II receptors leading to transphosphorylation of the type I receptor. The type I kinase activates proteins which migrate to the nucleus and together with other proteins regulate expression of target genes. GDF9, BMP15, BMP4, and BMp7 all use BMP2 as a binding receptor (27). BMP15 signals through interaction of BMPR1B and BMPR2 activating the SMAD1/5/8 pathway (28). Consequently, BMP proteins appear to interact with a limited number of receptors to activate two downstream Smad pathways. Moreover, several high-affinity binding proteins including follistatin, noggin, and gremlin antagonize BMP signaling (29). How granulosa cells and other cell types in the ovary differentiate between signals from multiple ligands in this pathway remains unclear.

Species Differences in BMP Signaling

There are both similarities and important differences between species in regulation in BMP signaling and consequences of gene knockouts in this pathway. Both mice and sheep with knockout mutations in GDF9 are infertile. In contrast to the dramatic consequences of homozygous mutations of BMP15 in sheep, targeted deletion of the second exon of Bmp15 in the mouse has little effect on folliculogenesis (30). However, the knockout mice are sub-fertile due to defects in the ovulation process and early embryo development. Reasons for the difference are not clear, but may relate to the relative importance of BMP15 and GDF9 in regulating follicle development in the different species. Another possibility is differential effects of the gene disruptions on processing and formation of GDF9/BMP15 homo- and heterodimers (22). BMP15 seems essential for human fertility (31). Two sisters with hypergonadotropic ovarian failure due to ovarian dysgenesis carry a non-conservative amino acid substitution in the promoter region of BMP15 (tyr235Cys) which acts in a dominant negative fashion by altering processing of BMP15.

CLINICAL REPRODUCTIVE ENDOCRINOLOGY

Stimulation Protocols

The first IVF baby was born as a result of an oocyte picked up in a natural cycle. However, the success rate of this protocol was very low, and the Monash group first reported large numbers of eggs and improved pregnancy rates using a stimulation protocol of clomiphene citrate and human menopausal gonadotropin (HMG) together (32). Several other regimens using these two medicines were subsequently reported. The common problems with these protocols were that endogenous gonadotropins led to premature luteinization in 30–40% of the cases, and, in others, ovulation occurred at an inconvenient time of the day. The major step in simplifying IVF induction of ovulation protocols and preventing these unwanted phenomena came with the introduction of GnRH agonists. GnRH analogs were created by a series of modifications in the GnRH molecule that led to the availability of new agonists and antagonists. The agonists initially enhance gonadotropin released from the pituitary but, with continuing administration, caused downregulation of the pituitary and reduced LH and FSH secretion for as long as the analog was given. This effect was a powerful tool with which to control the stimulated IVF cycle. GnRH antagonists are now available for routine clinical use (see Chapter 5).

Initially, the use of GnRH agonists was confined to women with a history of unsatisfactory response to other stimulation protocols, a premature LH surge, or elevated plasma LH and FSH levels (33,34). There are now many reports advocating their use in all IVF cycles including those women

with normal basal gonadotropins (35–37). Tan et al. (38) have reported life table analysis of conception and live birth rates in 3000 women undergoing IVF-ET with and without different regimens of the GnRH agonist buserelin. They found that the cumulative conception rate and cumulative live birth rate were significantly higher in those patients treated with a downregulation regimen. The mechanism by which GnRH analogs improve follicular response is not yet known. Palermo et al. (34) reported improvement in synchronization of follicular development leading to a larger cohort of developing follicles, whereas others related it to the longer gonadotropin stimulation in the GnRH analog cycles.

Urinary gonadotropin preparation, HMG, which contains FSH and LH, was used since 1980 for ovarian stimulation for IVF (32). In 1991, a highly purified FSH preparation was introduced, suggesting that endogenous LH was sufficient for satisfactory folliculogenesis (39). A meta-analysis of randomized trials of FSH versus HMG performed on 599 patients indicated that the use of FSH was associated with a significantly higher clinical pregnancy rate than HMG (40).

Further meta-analyses by NICE have addressed cost-effective studies relating to gonadotropins. The National Collaborating Centre for Women's and Children's Health found that HMG, urinary FSH, and recombinant FSH were equally effective in achieving a live birth when used following pituitary downregulation as part of IVF treatment. NICE recommended that consideration should be given to minimizing cost when prescribing. In the United Kingdom, use of urinary FSH products could represent a potential cost saving of £15 million/year.

Typical protocols of ovarian stimulation in a typical IVF program are:

1. GnRH agonist/FSH—long downregulation protocol.
2. GnRH agonist/FSH—short ("boost" or "flare-up") protocol.
3. Clomiphene citrate/HMG.

The objective of the protocols is to obtain a cohort of codominant follicles in both ovaries with adequate E2 levels suitable for oocyte retrieval on days 11–15.

GnRH Agonist/FSH—Long Downregulation Protocol

This protocol (Fig. 2) is currently the protocol of choice for first cycle patients and previously normal or high responders in many IVF programs. A GnRH agonist [Leuprolide, 1 mg SC, Buserelin 600 μg SC; or Nafarelin acetate (Synarel)], 400 μg nasal spray, is given for 10–14 days before starting gonadotropin treatment ("long-protocol"), and may be commenced in the mid-luteal phase of the previous cycle or on day 2 of the cycle. We, in addition to other researcher found that pituitary suppression was more effective when the therapy commenced in the mid-luteal phase of the previous cycle

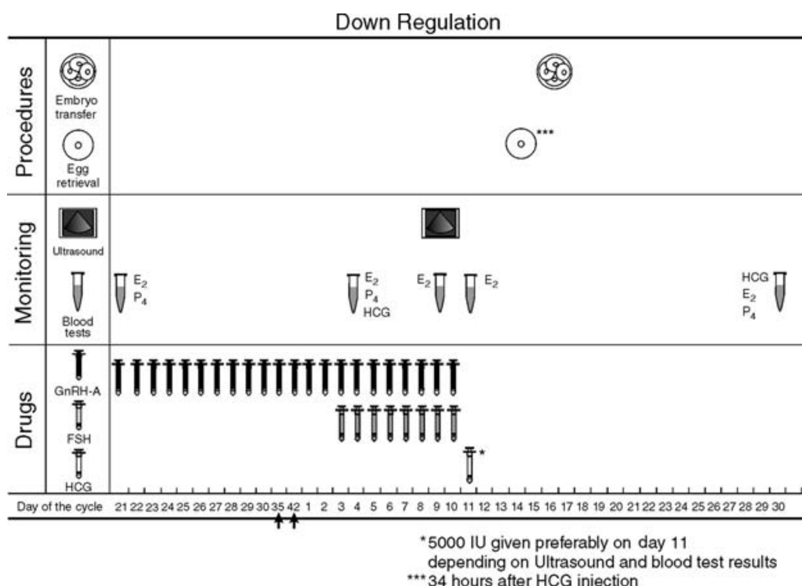

Figure 2 A representation of the luteal-phase commencement long downregulation protocol using FSH for ovarian stimulation for assisted reproductive technology. *Abbreviations*: hCG, human chorionic gonadotropin; GnRH, gonadotrophin-releasing hormone; FSH, follicle-stimulating hormone.

rather than in the early follicular phase of the treatment (35). Urbancsek and Witthaus (41) found that clinical pregnancy and live birth rates were better when a GnRH agonist was started in the mid-luteal phase rather than in the early follicular phase. Another advantage in commencing the analog in the mid-luteal phase was the decreased occurrence of ovarian cysts compared with commencing the GnRH agonist at the beginning of the follicular phase (42). These cysts, especially if functional, should be aspirated before commencing gonadotropin treatment.

Suggested criteria for pituitary–ovarian downregulation are E2 levels <180 pmol/L, LH levels <2 IU/L, and P4 levels <2 nmol/L. FSH commences after achievement of adequate downregulation and continues by daily injections according to individual endocrine and ovarian ultrasonic response until the day before the human chorionic gonadotropin (hCG) injection. A 5000 IU IM injection is given when the follicular cohort consists of at least three follicles of 17–20 mm in diameter and serum E2 level is appropriately rising. Egg pick-up follows 36 hr later.

Disadvantages of this protocol include a greater consumption of GnRH agonist and FSH and, therefore, greater patient cost, as well as the uncertainty of possible pregnancy upon initiation of GnRH agonist treatment if the GnRH analog is started on day 21 of the previous cycle.

Table 1 Follicle-Stimulating Hormone Starting Dose in In Vitro Fertilization Ovarian Stimulation Protocols

Patient group	Dose (IU/day)
First cycle <37 year old	150
First cycle with baseline scan suggesting PCOS	112
First cycle patient 37–39 year old	225
First cycle patient ≥40 year old	300
Previous normal response (≥5 oocytes)	150
Previous OHSS	75
Previous poor response (serum E2, 5th percentile)	450
BMI >30 kg/m^2 (PCOS excluded)	Increase by 75
Severe endometriosis	Increase by 75

Abbreviations: PCOS, polycystic ovarian syndrome; OHSS, ovarian hyperstimulation syndrome; BMI, body mass index.

For this reason, we ask our patients to avoid unprotected sexual intercourse in the cycle where GnRH analog treatment begins. However, published data suggest that pregnancy outcome is not adversely affected by GnRH agonist administration in the luteal phase of the conception cycle (43). Luteal support is important in these patients to avoid early abortion.

Patient age and follicular-phase serum FSH values are two useful predictors of gonadotropin dosage. FSH starting dose for the different patient groups in our program are listed in Table 1. The duration and daily doses of FSH are adjusted according to individual patient age and response as judged by serum E2 levels and follicular number and size in an ultrasound scan of the ovary.

The National Collaborating Centre for Women's and Children's Health have reviewed ovarian monitoring during gonadotropin therapy for IVF. NICE found that serum estradiol monitoring provided no additional information compared with ovarian ultrasound and, therefore, do not recommend it. This is different to the above guidelines for basic ovarian stimulation and monitoring. We are comfortable with this contradiction. We consider that serum E2 measurement does assist inexperienced units towards effective multifollicular ovulation induction. It also helps minimize ovarian hyperstimulation syndrome (OHSS). The above endocrinology guidelines may assist those units less experienced in IVF stimulation and monitoring.

GnRH Agonist/FSH—Short Boost Protocol

This protocol (Fig. 3) is typically reserved for poor responders, namely patients with day 3 FSH >10 IU/L and patients with a previous IVF cycle in which they have been cancelled or less than three oocytes were retrieved ("poor responders") or women who previously had a live delivery with a boost cycle. This protocol "boosts" the exogenous gonadotropin injected

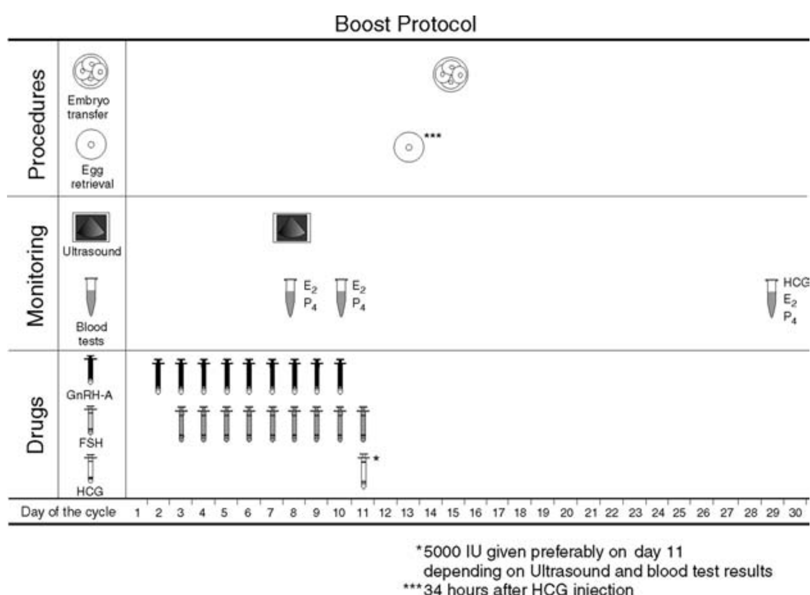

Figure 3 A diagram of the procedures, monitoring, and medicines used for short or Boost protocol commencing on day 2 of the menstrual cycle. *Abbreviations*: GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotrophin.

into the patient by a rise in serum concentration of her endogenous FSH. In this protocol, the GnRH agonist administration begins on day 2 of the cycle and FSH starts on day 3 of the cycle. Both medicines continue on the same regimen as the downregulation protocol. hCG administration criteria and dosage are the same as for the downregulation protocol.

The most important disadvantage of this protocol is the high P4 levels during the early follicular phase. This is most probably caused by rescue of the preceding corpus luteum. If the serum P4 is >6 nmol/L in the late follicular phase, we recommend cancellation of the cycle. NICE guidelines confirm a decreased clinical pregnancy rate per cycle using the short protocol compared with the long GnRH agonist protocol. The relative cost of agonists in the various regimens required for IVF require further economic studies to ascertain whether there is any true difference in cost.

Clomiphene Citrate and FSH/HMG Protocol

This protocol used to be the preferred protocol in most IVF units prior to development of GnRH analogs. As mentioned, it was abandoned in our program as the dominant superovulatory regimen because of the high cancellation rate due to untimely LH surges and some reports of the possible adverse effect of clomiphene citrate on the endometrium. In our unit, this

protocol is used only for patients who have responded well (>5 oocytes retrieved) to clomiphene citrate and HMG in the past and who did not respond well to the downregulation or boost protocols, or to patients who did not want to use GnRH agonists and who previously responded well to the combination of clomiphene citrate and FSH/HMG.

In this protocol, clomiphene citrate 100 mg/day commences on day 2 of the cycle and proceeds for 5 days. HMG begins on day 3 and continues, as do the other protocols, until 1 day before the hCG injection. hCG (5000 IU in IM injections) is given when the estimated leading follicle is 18–19 mm in diameter with serum E2 levels >1800 pmol/L.

Natural Cycle

In natural cycle IVF, one aspirates the oocyte that has matured during the natural follicular growth just after the natural LH surge. In the first successful IVF-ET treatment described by Edwards and Steptoe in 1978, the mature oocyte was retrieved from such a natural cycle. However, the success rate of this protocol was low and with some exceptions it is currently not in use in most IVF units (see Chapter 8).

Cycle Monitoring

NICE guidelines recommend ultrasound monitoring only to measure follicle size and follicle number for patient management during multi follicular folliculogenesis for IVF. Specifically, serum E2 monitoring is not recommended. We have minimized the use of serum E2, P4, and LH measurements during cycle monitoring in our IVF program and still find these helpful in our IVF program for basic ovarian stimulation. We do accept that experienced IVF units may have no difficulty in preventing ovarian hyperstimulation in all their patients without using endocrine measurements. However, we find OHSS still unpredictable to prevent and do recommend serum endocrinology to assist clinical IVF decision-making and minimize OHSS risk. That advice is summarized below.

As clinical knowledge about the nature of IVF cycles accumulates, cycle monitoring has become easier and less complicated. In our IVF program, cycle monitoring initially comprised daily serum E2, LH, and P4 estimations and ovarian ultrasound determinations. Currently, cycle monitoring consists of blood sampling for E2 levels at days 7 and 9 of the cycle and ultrasound scan of the ovary only on day 9. According to the results on day 7, a decision about FSH dose is made using the following criteria:

1. 1000 > E2 < 4500 pmol/L—continue with the same dose.
2. E2 < 1000 pmol/L—increase FSH dose [except polycystic ovarian syndrome (PCOS)].
3. E2 > 4500 pmol—decrease FSH dose.

On the second visit (day 9), if six or more follicles are >16 mm and E2 is >4000 pmol/L, hCG will be given the following day, followed by oocyte retrieval 36 hr later. If the criteria for hCG administration have not been met, a third blood sample is necessary to determine the right time for hCG injection and egg pick-up.

About 10% of patients on the IVF program are discharged before egg pick-up. Cancellation criteria in our unit are:

1. Serum P4 >6 nmol/L on day 9.
2. Fewer than three follicles >16 mm and E2 less than 3000 pmol/L on day 9 or 10.
3. Serum LH >30 IU/L during a clomiphene citrate cycle.
4. Abnormal finding by ultrasonography.
5. Falling serum E2 levels despite increasing FSH dose,
6. Serum E2 >25,000 pmol/L.

It is uncertain why all women with regular menstrual cycles do not respond to ovarian hyperstimulation protocols in a satisfactory fashion. We investigated spontaneous menstrual cycles after failure of multiple IVF folliculogenesis in 131 consecutive cycles. Thirty-six percent of the cycles were endocrinologically normal, whereas the rest had a range of endocrine abnormalities including low luteal phase P4, premature P4 elevation, occult ovarian failure, and hyperprolactinemia.

Although the importance of plasma P4 levels at the time of hCG administration is still controversial, most authors concluded that the subtle P4 elevation during GnRH agonist/FSH ovulation induction for IVF/ET does not predict IVF outcome (44). We do not measure serum P4 in down-regulation/FSH cycles. Repetition of the same superovulation regimen after inadequate stimulation in an IVF program results in improved folliculogenesis in only 10% of our patients. A review of patient history and the previous response to IVF folliculogenesis is therefore necessary before a decision is made about the next stimulation cycle protocol.

POLYCYSTIC OVARIAN SYNDROME

PCOS is the most common cause of anovulatory infertility. This syndrome was first described by Stein and Leventhal in 1935. The PCOS Rotterdam Consensus Conference recently defined PCOS as a clinical syndrome comprising any two of the three features: amenorrhea or oligomenorrhea; clinical or biochemical hyperandrogenism, and bilateral polycystic ovaries on ultrasound (45). Although no single biochemical test is diagnostic of PCOS, most patients showed a characteristic ovarian ultrasonographic appearance, namely the presence of > 10 follicles between 2 and 10 mm in diameter. On the other hand, ultrasonographically identified polycystic ovaries are a common finding in apparently normal women, with a prevalence of

20–23% (46,47). On careful review, menstrual irregularities and evidence of hyperandrogenism are frequently associated with the presence of polycystic ovaries in these apparently healthy women (48).

Infertile women with PCOS represent a difficult therapeutic problem for assisted reproductive techniques because they have a higher incidence of OHSS (refer following section) (49). On transvaginal ultrasound scanning, the prevalence of polycystic ovaries has been reported to be 25–33% in an assisted reproduction treatment population (50). These data underline the fact that polycystic ovaries are prevalent in infertile women who often lack the clinical characteristics of classical PCOS.

It has been proposed that a dysfunction of cytochrome P-450c 17α in PCOS leads to an increased 17-hydroxyprogesterone (17-OHP) response to a GnRH agonist-induced gonadotrophin rise (51). We investigated 106 IVF patients undergoing a boost stimulation regimen by correlating the ovarian ultrasound pattern with serum testosterone, 17-OHP, androstenedione, and estradiol responses and with the clinical outcome. There was a significantly higher prevalence of ovarian hyperandrogenism (serum testosterone >0.5 nmol/L after dexamethasone administration) in patients with polycystic ovaries (23%) compared with normal ovaries (7%). Patients with polycystic ovaries had approximately double the 17-OHP and estradiol responses to GnRH agonist. The number of oocytes retrieved was positively correlated with the estradiol responses. Although there was no difference in the total amount of FSH used between the patients with polycystic and normal ovaries, the median peak estradiol concentration was 1.6 times greater and the oocyte yield 2.3 times greater in patients with polycystic ovaries. The overall pregnancy rate per transfer was 32% and did not differ between patients with or without polycystic ovaries. We concluded from this study that the presence of polycystic ovaries on a vaginal ultrasound scan was the single most important marker for ovarian abnormality in an assisted reproduction patient population. Detection of polycystic ovaries predicts a subset of patients with abnormal ovarian androgen metabolism, exaggerated 17-OHP and estradiol responses to a GnRH agonist, and a higher oocyte yield. The ultrasonographic changes characteristic of PCO should be sought in all women undergoing assisted reproduction. In such cases, the ovarian-stimulating protocol is modified by reducing FSH starting dose (Table 1) and careful follow-up of serum E2 levels and follicular number and size.

IVF-ET is an effective therapy for PCOS patients who are refractory to ovulation induction in vivo even in the absence of other associated infertility factors (52). However, laparoscopic diathermy of the ovarian surface and hilum should precede an IVF attempt in these patients, as this procedure can reduce serum androgen concentration and normalize ovarian function. Following this procedure, spontaneous ovulation rate is about 90% and conception rates are 40–70% (53). Thus, many authors recommend

this operation even before gonadotrophin treatment (54). Furthermore, it should be considered for patients who had difficulties in multifolliculogenesis or had severe OHSS after IVF treatment (refer following section).

The overall conception rates per cycle in PCOS patients compared favorably to conception rates in patients with other infertility diagnoses (55–58). The use of GnRH agonist is associated with a significant reduction in the incidence of pregnancy loss and improvement in the cumulative pregnancy rates (58). Salat-Baroux et al. (55) found that the longer the period of pituitary desensitization (the long protocol vs. shorter ones), the lower the levels of circulating androgens. The current protocol for PCOS patients in Monash IVF is the long downregulation protocol with starting FSH dosage of 112 IU/day.

OVARIAN HYPERSTIMULATION SYNDROME

OHSS is the major serious and potentially life-threatening complication of ovulation induction in IVF-ET treatment. It is characterized by transudation of protein-rich fluid from the vascular space into the peritoneal cavity and to a less extent, pleural and pericardial cavities. The basic pathophysiologic event in OHSS is an acute increase in capillary permeability; however, the exact factors responsible for this phenomenon have, until recently, not been clear.

Because intensity of the OHSS is related to the degree of ovarian response to ovulation induction therapy, OHSS is probably an exaggeration of normal ovarian physiology. Part of the angiogenic response, which occurs in the follicle at the time of ovulation, is increased vascular permeability VEGF. VEGF stimulates endothelial cell mitogenesis and renders capillaries highly permeable to high-molecular-weight protein (59). VEGF has been identified in rat (60) and primate ovaries predominantly after the LH surge. Luteal-phase treatment with GnRH agonist, to suppress LH secretion, decreased VEGF messenger-RNA expression, implying such expression is dependent on LH. We first reported the role of VEGF in OHSS (61). We have demonstrated that VEGF is the major capillary permeability factor in OHSS ascites. Although other capillary permeability factors may not have been detected, 70% of the capillary permeability activity in OHSS ascites was neutralized by recombinant human VEGF antiserum. Abramov et al. (62) subsequently followed the kinetics of VEGF in the plasma of seven patients with severe OHSS from the time of admission to the hospital until clinical resolution. They found that compared with normo-responders, IVF patients suffering OHSS had VEGF plasma levels which were significantly higher and correlated to the clinical picture. Ascitic fluid obtained from OHSS patients contained high VEGF levels.

The incidence of OHSS after induction of ovulation varies between 1% and 30%, as reported in various publications. This variation is probably due

to the difference in the definition of OHSS. OHSS is classically divided into three categories:

1. Mild OHSS, with high serum E2 levels, mild abdominal distension, and large ovaries of about 5 cm due to the presence of multiple follicular and corpus luteal cysts.
2. Moderate OHSS, characterized by more abdominal distension, mild ascites, and gastrointestinal symptoms such as nausea, vomiting, and less frequently, diarrhea, some gain in weight, and ovaries enlarged up to 12 cm.
3. Severe OHSS which can be a life-threatening situation, characterized by pronounced abdominal distension, ascites, pleural effusion, hemoconcentration, electrolyte imbalance, oliguria/anuria, and sometimes disseminated intravascular coagulation and hypovolemic shock. The ovaries are enlarged to more than 12 cm in diameter.

The treatment of OHSS is conservative. Bed rest and symptomatic relief are usually sufficient for mild and moderate OHSS. In mild cases, symptoms subside usually within a few days, whereas in moderate cases, symptoms can require up to 3 wk to subside. When pregnancy occurs, OHSS will last longer.

Severe OHSS can be life-threatening and patients, therefore, should be hospitalized and monitored closely. Patients are put on bed rest; daily body weight and fluid balance monitoring are necessary; hematocrit, coagulation and kidney functions, serum electrolyte, and albumin studies are obtained daily, as well as ultrasound scan of the pelvis and chest; oxygen saturation measurements (using an oximeter while breathing air and if less than 85% using blood gases) should be performed if dyspnoea is notified; and pelvic examination should be avoided because of the fragility of the enlarged ovaries and ovarian surgery is relatively contraindicated.

Hypovolemia and hemoconcentration need immediate corrections. When necessary, intravenous crystalloid infusion or plasma expanders are given to maintain central venous pressure. Replacement may require central venous pressure monitoring. Diuretics should not be given with, as the fluids in the abdominal and thoracic cavity are not responsive to diuretics and the further intravascular depletion can cause hypotension, shock, and thrombosis. Administration of low-dose heparin to prevent thromboembolism needs to be balanced against the risk of ovarian bleeding, possible need for paracentesis, and the possibility of heparian-induced thrombocytopenia. Most of our hospitalized patients are commenced on heparin 5000 U $\times$ 2/day subcutaneously on admission and reviewed after 24 hr. Heparin should be continued until discharge from the hospital if the patient has risk factors or past history of thrombosis, family history of thromboembolism, smoking, obesity, or varicose veins. Heparin should also be continued if there is hemoconcentration, tense ascites with inferior vena cava (IVC) compression, and late presentation with possible

multiple pregnancies. Abdominal paracentesis under ultrasonographic guidance is recommended where there are ascites with IVC compression and edema of the lower limbs. Indomethacin has been shown to have beneficial effects on hyperstimulation syndrome, but its safety in early pregnancy is unknown (63).

The acute clinical manifestation of the severe form of OHSS usually disappears within several days if there are no complications. If pregnancy does not occur, the ovarian enlargement subsides usually within 30–40 days. If, however, pregnancy occurs, the high serum E2 levels will continue to the end of the first trimester.

Prevention of OHSS is vital. Low body mass index and PCOS are two clinical predictors and gonadotropin dosage should be reduced in such patients. When OHSS is suspected before hCG injection (as when serum E2 >25,000 nmol/L), hCG should be withheld. If, however, OHSS is diagnosed after oocyte retrieval, embryos should be frozen in order to avoid conception and replaced later in a natural cycle (64) or with estrogen/progesterone therapy. This approach also allows GnRH agonist administration to be renewed immediately after oocyte retrieval and maintained until the onset of subsequent menstruation, further reducing the risk of significant OHSS (65). We use hCG at a dose of 5000 IU rather than 10,000 IU for final oocyte maturation before retrieval in all our IVF patients to help prevent OHSS.

In 1993, Asch et al. (66) claimed that prevention of severe OHSS was possible by administration of intravenous albumin at a dose of 5% in ringer lactate in doses of 500 ml during oocytes retrieval and 500 ml immediately thereafter. Subsequent prospective randomized trials by Shoham et al. (67), Shalev et al. (68), and Shaker et al. (69) all showed that the frequency and degree of OHSS developed in patients treated with intravenous albumin were not different than in control patients. However, the pregnancy rate was significantly higher in patients who had all embryos cryopreserved to be transferred subsequently in hormonally manipulated cycle than in patients treated with albumin and fresh ET.

POOR RESPONDERS

Poor responders to exogenous gonadotropins require markedly increased quantities of gonadotropins to obtain multifollicular development. Moreover, the number of oocytes, fertilization rates, embryo quality, and pregnancy rates are all decreased in this patient population. The incidence of poor response varies from 5% to 18% of unselected IVF patients (70). Poor response to gonadotropins is a result of diminished ovarian reserve. The most important cause is advanced female age. An age-related decline in fecundity, from 35 years in particular, has been observed in IVF. Other causes are prior chemotherapy, irradiation, ovarian surgery, severe endometriosis, autoimmune disease, or idiopathic. Some patients present with unexplained infertility and regular ovulatory cycles.

It is ideal to identify these patients before initiation of hormonal treatment for IVF. In this way, the patients can be counseled concerning the lower chances for pregnancy and stimulating protocols can be modified to lower the risk of cancellation and improve pregnancy rates. In 1987, Navot et al. (71) described the clomiphene citrate challenge test, a test measuring serum FSH levels before (day 3) and after day 10, the administration of 100 mg of clomiphene citrate on cycle days 5–9. They found that patients with an exaggerated FSH response, despite normal E2 response, had diminished ovarian reserve. They suggested that diminished capacity of the granulosa cells to secrete inhibin could explain the discrepancy between E2 and FSH response. This test was found to be a reliable method for predicting IVF-ET outcome. Later, several studies have shown that elevated day 3 serum concentrations of FSH (<6.5–15 IU/L) and/or E2 (<80 pg/ml) have poor pregnancy outcomes with fertility therapy (72). Martin et al. (73) evaluated 1868 cycles and found that no pregnancies occurred if day 3 FSH was >20 IU/L at least in two cycles, but if it happened just once, the pregnancy rate was 5.6%. Recently, Seifer et al. (74) found that women with low day 3 serum inhibin B concentrations (<45 pg/ml) demonstrated a poorer response to ovulation induction and IVF-ET treatment relative to women with high day 3 inhibin B.

ORAL CONTRACEPTIVE PRETREATMENT

The role of GnRH antagonists in human IVF is discussed in Chapter 5. Oral contraceptive pre-treatment for ovarian stimulation in a GnRH agonist or a GnRH antagonist cycle has been recently investigated. Oral contraceptive scheduling of a GnRH agonist or a GnRH antagonist protocol results in follicular growth and hormone profile are similar to those observed in GnRH agonist protocols. The number of premature LH rises remains low. Similar numbers of oocytes and high-quality embryos are obtained. This is significant because the use of the oral contraceptive pre-treatment method significantly improves scheduling in a typical IVF program operating Monday to Friday. The greater convenience of oral contraceptive pre-treatment scheduling appears off-set by the need for longer stimulation protocols and more FSH than with a nonschedule regimen.

OVARIAN STIMULATION AND OVARIAN CANCER

Concern has been expressed that exposure to fertility drugs might be associated with a risk of ovarian cancer. In particular, pooled analysis of three case-controlled studies by Whittemore et al. (75) suggested an odds ratio of 2.8 (95% CI 1.3–6.1) for invasive ovarian cancer infertile women treated with fertility drugs compared with women with no diagnosis of infertility or infertility drug treatment. This study, suggesting an apparent trebling

of risk in infertile women treated with fertility drugs, has been controversial and widely criticized for various biases in the study design.

In 1994, Rossing et al. undertook a study that yielded the best data, to this time, examining the role of clomiphene citrate and ovarian cancer. Rossing et al. used record linkage with a population-based cancer register. They identified an increased incidence of ovarian cancer comprising both borderline malignant tumors and invasive disease. They found a standardized incidence rate (SIR) of 2.5 with 95% confidence intervals (1.3–4.7) in a cohort of infertile women compared with age-standardized general population rates (76). This, of course, is a similar rate of increase found by Whittemore et al. in the previously mentioned study. In the Rossing data, an increased relative risk was also found in women who had been treated with clomiphene citrate for more than 1 year when compared with infertile women who had not taken clomiphene citrate. This increased risk of ovarian cancer was seen regardless of whether the patients did or did not have diagnosed ovarian abnormalities and were treated with clomiphene citrate. There was no increased risk when patients were prescribed clomiphene citrate for less than 1 year.

There are significant clinical differences between the prescription of clomiphene citrate to a U.S. population of women compared with the administration of clomiphene citrate in other parts of the world. In Australia, e.g., clomiphene citrate is only available on prescription from specialist gynecologists and obstetricians rather than from general practitioners as in the United States. Furthermore, clomiphene citrate can only be prescribed in Australia for patients with polycystic ovary syndrome or related disorders and not for women who are infertile but who are spontaneously ovulating. Moreover, our general advice is to use clomiphene citrate in order to achieve four to six ovulatory cycles but not more in the treatment of an infertile woman with polycystic ovary syndrome and chronic anovulation. The use of clomiphene citrate for more than 1 year in an infertile woman would be highly usual and not recommended in our experience.

Previous studies of cancer after infertility have been limited by low statistical power and difficulties in distinguishing possible effects of fertility drug exposure from the underlying ovulation disorder they were used to treat. We have completed a large study looking at the incidence of breast and ovarian cancer after infertility and IVF (77). The great majority of these patients received HMG or ovarian stimulation for IVF. In this study, we examined the incidence of breast and ovarian cancer and a cohort of 10,358 women referred for IVF treatment in Victoria, Australia, between 1978 and 1992.

The exposed group ($n = 5564$) had ovarian stimulation to induce multiple folliculogenesis for IVF. The unexposed group ($n = 4794$) had been referred for IVF but were untreated or had a natural cycle treatment without ovarian stimulation. The duration of follow-up in both groups ranged from 1 to 15 years.

Cases of cancer were determined by record linkage with data from population-based cancer registries. We observed 34 cases of invasive breast cancer and six cases of invasive ovarian cancer. A comparison with the expected numbers derived by applying age-standardized general population rates to the cohort gave SIR for breast cancer of 0.89 (95% CI 0.62–1.56) in the unexposed group. For ovarian cancer, the SIRs were 1.70 (0.55–5.27) and 1.62 (0.52–5.02), respectively. In this large study, we were able to look at the rates of all cancers in these two groups of individuals and not only their rates of breast and ovarian cancers. The rates of all cancers in these two groups of women were not significantly different from the general population rates in Victoria, Australia.

We found that the risk of cancer of the body of the uterus, particularly endometrial adenocarcinoma, was increased in both these groups of infertile women combined [SIR 2.84 (1.18–6.18)]. Women with unexplained infertility, regardless of whether they had gonadotropin treatment for IVF or not, also had a significantly increased risk of ovarian cancer [RR = 19.19 (2.23–165.0)] and cancer of the uterus [RR = 6.34 (1.06–38.0)] compared with women with known causes of infertility.

This relatively short-term follow-up of patients after HMG treatment for IVF indicates that ovarian stimulation with gonadotropins is not associated with any increased risk of breast cancer. We also found no significant increased risk of ovarian cancer after ovarian stimulation for IVF with gonadotropins. However, the small number of cases limits the conclusions that can be drawn. Longer-term follow-up of large cohorts of women who have been prescribed gonadotropins for ovulation induction or IVF programs will be necessary. NICE guidelines suggest that medical practitioners should confine the use of ovulation induction agents to the lowest effective dose and shortest duration of use (78).

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REFERENCES

1. Vermesh M, Kletzky OA. Longitudinal evaluation of the luteal phase and its transition into the follicular phase. J Clin Endocrinol Metab 1987; 65:653.
2. Gorgeon A, Lefevre B. Evaluation of the largest healthy and atretic follicles during the human menstrual cycle. J Reprod Fertil 1983; 69:497.
3. Pache TD, Wladimoroff JW, de Jong FH, Hop WC, Fauser BCJM. Growth patterns of non-dominant ovarian follicles during the normal menstrual cycle. Fertil Steril 1990; 54:638.

4. Tsafriri A, Adashi EY. Local nonsteroidal regulators of ovarian function. In: The Physiology of Reproduction. Vol. 1. 2nd ed. New York: Raven Press, 1994:817.
5. Findlay JK. An update on the roles of inhibin, activin and follistatin as local regulators of folliculogenesis. Biol Reprod 1993; 48:15.
6. Ying SY, Becker A, Long N, Uneno N, Guillemin LR. Inhibin and G type transforming growth factor have opposite modulating effects on the follicle-stimulating hormone (FSH) induced aromatase activity of cultured rat granulosa cells. Biochem Biophys Res Commun 1986; 136:969.
7. Di Simone P, Lanzome A, Petralgia F, Ronsville E, Caruso A, Mancuso S. Effects of activin-A on progesterone synthesis in human luteal cells. Fertil Steril 1994; 62:1157.
8. Hutchinson LA, Findlay AK, de Vos FC, Robertson DM. Effect of bovine inhibin, transforming growth factor-beta and bovine activin-A in granulosa cell differentiation. Biochem Res Commun 1987; 146:1405.
9. Miro F, Hillier SG. Modulating of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. Endocrinology 1996; 137:464.
10. Woodruff TK, Mather JP. Inhibin, activin and the female reproductive axis. Annu Rev Physiol 1995; 57:219.
11. Xaio SY, Robertson DM, Findlay JK. Effects of activin and follicle-stimulating hormone (FSH) suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 1992; 131:1009.
12. Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznick AJ. Vascular endothelial growth factor mRNA expression in the primate ovary in relation to follicular growth and corpus luteum function. Endocrinology 1992; 2065:254.
13. Taymor ML. The regulation of follicular growth: some clinical implications in reproductive endocrinology. Fertil Steril 1996; 65:235.
14. Chandrasekher YA, Hutchinson JS, Zelinski-Wooten MB, Hess DL, Wolf DP, Stouffer RL. Initiation of periovulatory events in primate follicles using recombinant and native human luteinizing hormone to mimic the midcycle gonadotrophin surge. J Clin Endocrinol Metab 1994; 79:298.
15. Erickson GF, Kokka S, Rivier C. Activin causes premature superovulation. Endocrinology 1995; 136:4804.
16. Adashi EY. Immune modulation in the context of the ovulation process: a role for interleukine-1. Am J Reprod Immunol 1996; 35:190.
17. Tsafriri A. Ovulation as a tissue remodelling process: proteolysis and cumulus expansion. Adv Exp Med Biol 1995; 337:121.
18. Laitinen M, Vuojolainen K, Jaatinen R, et al. A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. Mech Dev 1998; 78:135–140.
19. McGrath SA, Esquela AF, Lee SJ. Oocyte-specific expression of growth/differentiation factor-9. Mol Endocrinol 1995; 9:131–136.
20. Otsuka F, Yao Z, Lee T, et al. Bone morphogenetic protein-15. J Biol Chem 2000; 275:39523–39528.
21. Erickson GF, Shimasaki S. The physiology of folliculogenesis: the role of novel growth factors. Fertil Steril 2001; 76:943–949.
22. Shimasaki S, Moore RK, Otsuka F, et al. The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 2004; 25:72–101.

23. Otsuka F, Moore RK, Shimasaki S. Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 2001; 276:32889–32895.
24. Massague J. TGF-β signal transduction. Ann Rev Biochem 1998; 67:753–791.
25. Shimasaki S, Zachow RJ, Li D, et al. A functional bone morphogenetic protein system in the ovary. Proc Natl Acad Sci USW 1999; 96:7282–7287.
26. Sebald W, Nickel J, Zhang JL, et al. Molecular recognition in bone morphogenetic protein (BMP)/receptor interaction. Biol Chem 2004; 385:697–710.
27. Vitt UA, Mazerbourg S, Klein C, et al. Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9. Biol Reprod 2002; 67:473–480.
28. Moore RK, Otsuka F, Shimasaki S. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 2003; 278:304–310.
29. Pangas SA, Jorgez CJ, Matzuk MM. Growth differentiation factor 9 regulates expression of the bone morphogenetic protein antagonist gremlin. J Biol Chem 2004; 279:32281–32286.
30. Yan C, Wang P, DeMayo J, et al. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 2001; 15:854–866.
31. Di Pasquale E, Beck-Peccoz P, Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet 2004; 75:106–111.
32. Trounson AO, Leeton JF, Wood EC, Webb J, Wood J. Pregnancies in humans by fertilisation in vitro and embryo transfer in the controlled ovulatory cycle. Science 1981; 212:616.
33. Edelstein MC, Brzyski RG, Jones GS, Oehninger S, Sieg SM, Muasher SJ. Ovarian stimulation for in vitro fertilization using pure follicle-stimulating hormone with and without gonadotrophin-releasing hormone 23 in high-responder patients. JIVET 1990; 7:172.
34. Palermo R, Amadeo G, Navot D, Rosenwaks Z, Cittandi E. Concomitant gonadotrophin-releasing hormone agonist and Metrodin treatment for the synchronized induction of multiple follicles. Fertil Steril 1988; 49:290.
35. MacLachlan V, Besanko M, O'Shea FF, et al. A controlled study of luteinizing hormone-releasing hormone agonist (Buserelin) for induction of folliculogenesis before in vitro fertilization. N Engl J Med 1989; 320:1233.
36. Meldrum DR, Wilsot A, Hamilton F, Gutlay AL, Kempton W, Huynh D. Routine pituitary suppression with leuprolide before ovarian stimulation for oocyte retrieval. Fertil Steril 1989; 51:455.
37. Meldrum DR, Wisot A, Hamilton F, Cutlay AL, Huynh Kempton W. Timing of initiation and dose schedule of leuprolide influence the time course of ovarian suppression. Fertil Steril 1988; 50:400.
38. Tan SL, Royston P, Jackobs HS, Balan A. Cumulative conception and live birth rates after in vitro fertilization. Lancet 1992; 339:1390.
39. Chappel SC, Howles EC. Re-evaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. Hum Reprod 1991; 9:1206.
40. Daya S, Gunby J, Hughes EG, Collins JA, Sagle MA. Follicle stimulating hormone versus human menopausal gonadotropin for in vitro fertilization cycles: a meta-analysis. Fertil Steril 1995; 64:347.

41. Urbancsek J, Witthaus E. Midluteal buserelin is superior to early follicular phase buserelin in combined gonadotropin-releasing hormone analog and gonadotropin stimulation in in vitro fertilization. Fertil Steril 1996; 65:966.
42. Ben-Rafael Z, Bider D, Menashe Y, Maymon R, Zolti M, Mashiach S. Follicular and luteal cysts after treatment with gonadotrophin-releasing hormone analog for in vitro fertilization. Fertil Steril 1990; 53:1091.
43. Cahill DJ, Fountain SA, Fox R, Fleming CE, Brinsden PR, Hull MG. Outcome of inadvertent administration of a gonadotrophin-releasing hormone agonist (buserelin) in early pregnancy. Hum Reprod 1994; 9:337.
44. Bustillo M, Stern JJ, Coulam CB. Serum progesterone at the time of human chorionic gonadotrophin does not predict pregnancy in in vitro fertilization and embryo transfer. Hum Reprod 1995; 10:2862.
45. The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004; 81(1):19–25.
46. Polson DW, Adams J, Wadsworth J, Franks S. Polycystic ovaries—a common finding in normal women. Lancet 1988; 1:870.
47. Clayton RN, Ogden V, Hodgkinson J, et al. How common are polycystic ovaries in normal women and what is their significance for the fertility of the population? Clin Endocrinol 1992; 37:127.
48. Franks S. Polycystic ovary syndrome: a changing perspective. Clin Endocrinol 1989; 31:87.
49. MacDougall MJ, Tan SL, Jacobx HS. In vitro fertilization and the ovarian hyperstimulation syndrome. Hum Reprod 1992; 7:597.
50. Macdougall MJ, Tan SL, Balan A, Jacobs HS. A controlled study comparing patients with an without polycystic ovaries undergoing in vitro fertilization. Hum Reprod 1993; 8:233.
51. Rosenfield RL, Barnes RB, Cara JF, Lucky AW. Dysregulation of cytochrome P450c 17α as the cause of polycystic ovarian syndrome. Fertil Steril 1990; 53:785.
52. Buyalos RP, Lee CT. Polycystic ovary syndrome: pathophysiology and outcome with in vitro fertilization. Fertil Steril 1996; 65:1.
53. Naether OGN, Baukloh V, Fischer R, Kowalczykl T. Long-term follow-up in 206 infertility patients with polycystic ovarian syndrome after laparoscopic electrocautery of the ovarian surface. Hum Reprod 1994; 9:2342.
54. Farhi J, Soule S, Jacobs HS. Effect of laparoscopic ovarian electrocautery on ovarian response and outcome of treatment with gonadotropins in clomiphene citrate resistant patients with polycystic ovary syndrome. Fertil Steril 1995; 64:930.
55. Salat-Baroux J, Alvarez S, Antoine JM, et al. Results of IVF in the treatment of polycystic ovary disease. Hum Reprod 1988; 3:331.
56. Dor J, Shulman A, Levran D, Ben-Rafael Z, Rudak E, Mashiach S. The treatment of patients with polycystic ovarian syndrome by in vitro fertilization and embryo transfer: a comparison of results with those of patients with tubal infertility. Hum Reprod 1990; 5:816.
57. Urman B, Fluker MR, Yuen BH, Fleige-Zahradka BG, Ouves CG, Moon YS. The outcome of in vitro fertilization and embryo transfer in women with polycystic ovary syndrome failing to conceive after ovulation induction with exogenous gonadotropins. Fertil Steril 1992; 57:1269.

58. Homburg R, Berkowitz D, Levy T, Feldberg D, Ashkenazi J, Ben-Rafael Z. In vitro fertilization and embryo transfer for the treatment of infertility associated with polycystic ovary syndrome. Fertil Steril 1993; 60:858.
59. Ferrara N, Houck KA, Jukeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. J Cell Biol 1991; 47:211.
60. Phillips HS, Hains J, Leung DW, Ferrara N. Vascular endothelial cell growth factor is expressed in rat corpus luteum. Endocrinology 1990; 127:965.
61. McClure N, Healy DL, Rogers PAW, et al. Vascular endothelial growth factor as capilli permeability agent in ovarian hyperstimulation syndrome. Lancet 1994; 344:235.
62. Abramov Y, Barak V, Nisman B, Schenker JG. Vascular endothelial growth factor plasma levels correlate to the clinical picture in severe ovarian hyperstimulation syndrome. Fertil Steril 1997; 67:261.
63. Schenker JG, Winstein D. Ovarian hyperstimulation syndrome: a current survey. Fertil Steril 1978; 30:255.
64. Smitz J, Camus M, Devroey P, Erard P, Wisanto A, Van Steirteghem AC. Incidence of severe ovarian hyperstimulation syndrome after GnRH/HMG superovulation for in vitro fertilization. Hum Reprod 1990; 5:933.
65. Wu TCJ, Gelety TJ, Jih MH, Fournet N, Buyalos RP. Successful management of predicted severe ovarian hyperstimulation syndrome with gonadotropin-releasing hormone agonist. J Assist Reprod Genet 1992; 9:281.
66. Asch RH, Ivery G, Goldsman M, Fredrick JL, Stone SC, Balmaceda JP. The use of intravenous albumin in patients at high risk for severe ovarian hyperstimulation syndrome. Hum Reprod 1993; 8:1015.
67. Shoham Z, Weissman A, Barash A, Borenstein R, Schachter M, Insler V. Intravenous albumin for the prevention of severe ovarian hyperstimulation syndrome in an in vitro fertilization program: a prospective, randomized placebo-controlled study. Fertil Steril 1994; 62:137.
68. Shalev E, Gilacli Y, Matilsky M, Ben-Ami M. Decreased incidence of severe ovarian hyperstimulation syndrome in high risk in vitro fertilization patients receiving intravenous albumin: a prospective study. Hum Reprod 1995; 10:1373.
69. Shaker AG, Zosmer A, Dean N, Bekir JS, Jacobs HS, Tan SL. Comparison of intravenous albumin and transfer of fresh embryos with cryopreservation of all embryos for subsequent transfer in prevention of ovarian hyperstimulation syndrome. Fertil Steril 1996; 65:992.
70. Jenkins JM, Davies DW, Devonport H, et al. Comparison of "poor" responders with "good" responders using a standard buserelin/human menopausal gonadotropin regime for in vitro fertilization. Hum Reprod 1991; 6:918.
71. Navot D, Rosenwaks Z, Margalioth EJ. Prognostic assessment of female fecundity. Lancet 1987; 8560:645.
72. Muasher SJ, Oehninger S, Simonetti S, et al. The value of basal and/or stimulated serum gonadotropin levels in prediction of stimulation response and in vitro fertilization outcome. Fertil Steril 1988; 50:298.
73. Martin JSB, Nisker JA, Tummon IS, Daniel SAJ, Auckland JL, Feyles V. Future in vitro fertilization pregnancy potential of women with variably elevated day 3 follicle-stimulating hormone levels. Fertil Steril 1996; 65:1238.

74. Seifer DB, Lambert-Messerlian G, Hogan JW, Gardiner AC, Blazar AS, Berk CA. Day 3 serum inhibin-B is predictive of assisted reproductive technologies outcome. Fertil Steril 1997; 67:110.
75. Whittemore A, Harris R, Itnyre J. Collaborative Ovarian Cancer Group. Characteristics related to ovarian cancer risk: collaborative analysis of twelve US case–control studies. II. Invasive epithelial cancer in white women. Am J Epidemiol 1992; 136:1184.
76. Rossing MAS, Daling JR, Weiss NS, Moore DE, Self SG. Ovarian tumors in a cohort of infertile women. N Engl J Med 1994; 331:771.
77. Venn A, Watson L, Lumley J, Giles G, King C, Healy D. Breast and ovarian cancer incidence after infertility and in vitro fertilization. Lancet 1995; 346:995.
78. Fertility: assessment and treatment of people with fertility problems. 2004 Clinical guidelines. National Collaborating Centre for Women's & Children's Health, National Institute for Clinical Excellence, RCOG Press, 2004.

5

Gonadotropin-Releasing Hormone-Antagonist in Human In Vitro Fertilization

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INTRODUCTION

Gonadotropin-releasing hormone antagonists (GnRH-nt) available for clinical use are GnRH molecules with amino acid modifications in positions 1, 2, 3, 6, and 10. They are not associated with the histaminic-release effects of previous compounds (1). These compounds immediately block GnRH receptor in a competitive fashion (2). They decrease the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion within a period of eight hours. Inhibition of LH secretion is more important than FSH. This is probably due to the different forms of gonadotropin regulation, the prolonged FSH half-life, or the immunoactive and bioactive forms of FSH (3,4).

Administered during the follicular phase, GnRH-nt can prevent or interrupt LH surges (5). In addition, their use has been proposed in in vitro fertilization (IVF)–embryo transfer (ET) cycles to obtain results similar to those obtained with GnRH-a, however with the simplest protocol and fewer side effects (6).

Two different compounds are available: the Cetrorelix (Cetrotide®, formerly ASTA Medica, now Serono) and the Ganirelix (Antagon® or Orgalutran®, Organon). Two different protocols of administration (Fig. 1) have been proposed in the literature for using GnRH-nt in controlled

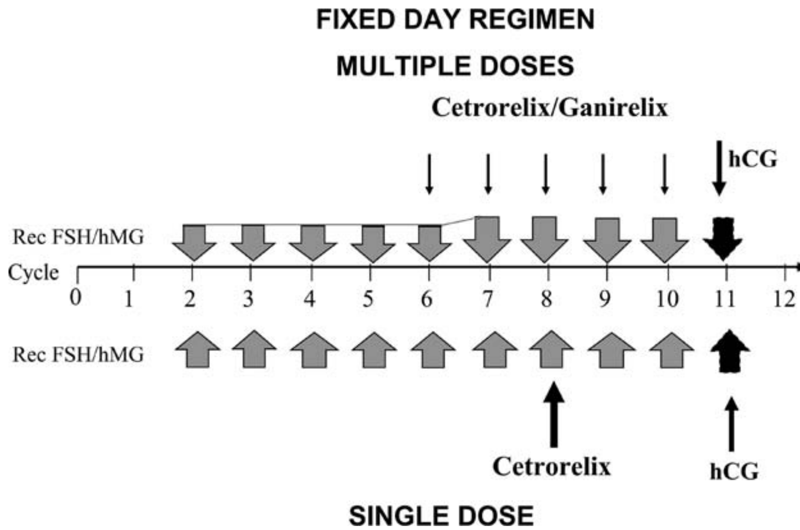

Figure 1 Gonadotropin-releasing hormone antagonist multiple- and single-dose protocols. Fixed day regimens. *Abbreviations*: FSH, follicle-stimulating hormone; hMG, human menopausal gonadotropin; hCG, human chorionic gonadotropin.

ovarian stimulation (COH). In the multiple-dose protocol, small doses (0.25 mg) of the GnRH-nt are injected in the middle of the follicular phase (7–9). In the single-dose protocol, a higher dose (3 mg) is injected during the late follicular phase, when the LH surge is most feared (10,11).

PHASE II DOSE-FINDING STUDIES

Single-Dose Protocol

In the first investigation with Cetrorelix, we simply reproduced the previously published Nal–Glu protocol consisting of two 5 mg injection 48 hr apart in the late follicular phase (12,13). We therefore proposed two administrations of 5 mg Cetrorelix 48 hour apart, the first injection being administered on stimulation day 7. We observed that the second injection was often unnecessary as hCG was given on the same day. We concluded also that the 5 mg dose induced a deep suppression of LH and that a lower dose should be tried (10). A single-dose protocol was designed where a single injection of 3 mg of the GnRH-nt is performed on stimulation day 7 (11).

To determine the minimal effective dose, we conducted a dose-finding study. We compared the use of 2 and 3 mg to investigate the "protection period," the time during which an LH surge is prevented after the antagonist administration. The IVF-ET results were strictly comparable between the

two doses and the 2 mg dose prevented LH surges for three days in all the patients. However, we observed that the suppression of LH tended to be reduced three days after the injection in the 2 mg dose and an LH surge was observed four days after the 2 mg Cetrorelix administration. The 3 mg dose was therefore selected as a safer choice, as a "protection period" of at least four days can be obtained (14). No LH surge was observed in all the patients treated with the 3 mg dose. In some patients, an LH rise (LH > 10 IU/L) was observed on the day of the antagonist administration. The Cetrorelix was able to prevent any further rise in LH, lowering immediately the LH levels and no surge was observed in these patients. Moreover, the interruption of LH rises does not seem to have a deleterious effect on IVF-ET results (15). The consumption of hMG was clearly reduced (24–30 hMG ampoules) as compared to the use of GnRH-a in the long protocol using a depot preparation (11).

The tolerance of the GnRH-nt Cetrorelix was excellent with only transient erythema at the injection site in 15% of the patients.

Multiple-dose

The two GnRH-nts (Cetrorelix or Ganirelix) were studied in order to achieve the best dose that blocks the premature LH rise and does not oversuppress the pituitary. Sommer et al. (16) were the first in describing the suppression of gonadotropin and estradiol secretion after 3 mg of Cetrorelix daily in normal cycling women. After that, the dose-finding studies generally initiated the gonadotropins (recombinant or urinary) on day 2 of menstrual cycle and the antagonist daily administration was initiated on stimulation day 6 (7–9,17). The risk for premature LH surge is higher after the sixth day of ovarian stimulation, and this day was chosen to initiate the antagonist injections (7). The authors of the different studies compared different doses of Cetrorelix or Ganirelix to achieve the best dose with most appropriate assisted reproductive technology (ART) results.

Comparing the Cetrorelix administration of three and 1 mg or 0.5 and 0.25 mg after day 6 of stimulation protocol, the authors showed that all patients had a decline on LH serum levels. The group of patients receiving 0.5 and 0.25 mg/day showed the best ART results in terms of pregnancy and implantation rates without the risk of a pituitary oversuppression which occurred with one and 3 mg (7,17). Another study compared the starting doses of 0.5, 0.25, and 0.1 mg/day (8). The authors demonstrated that patients receiving 0.5 or 0.25 mg/day during the follicular phase did not showed premature LH surge, evidenced by the lower LH serum levels. However, one out of seven patients with 0.1 mg/day protocol showed a premature LH rise with progesterone elevation and the 0.1 mg dose was therefore abandoned. The results were similar between the patients receiving 0.25 and 0.5 mg/day in terms of clinical pregnancy and implantation rates.

The group of investigators concluded that the minimal necessary and effective dose to prevent premature LH surge was 0.25 mg/day with Cetrorelix.

The Ganirelix Study Group (9) also investigated the minimal safe/effective dose to achieve good IVF results. This study also showed that, during the multiple dose protocol with Ganirelix, the minimal effective dose was 0.25 mg/day, inhibiting the premature LH secretion without compromising IVF results in stimulated cycles with recombinant FSH. This group of patients, receiving Ganirelix 0.25 mg/day had the highest vital pregnancy rate per transfer (40.3%) as the main clinical outcome if compared with the others doses (0.0625–2 mg).

Observing very low implantation rates in the groups of daily 1 or 2 mg, Kol et al. (18) analyzed the database from the Ganirelix dose-finding study (9) concerning the effect of GnRH-nt in freeze–thaw cycles. The authors concluded that high doses (1.0 and 2.0 mg/day) of Ganirelix did not affect the biologic potential of embryos to develop clinical pregnancy.

PHASE III RANDOMIZED CONTROLLED TRIALS AND OPEN STUDIES

Single-Dose Protocol

We have compared our single-dose protocol to the GnRH-a long protocol using a depot formula of Triptorelin in a prospective randomized study (6). A 3:1 randomization was selected including 115 patients in the Cetrorelix group and 36 in the agonist long protocol group. No difference was observed between the GnRH agonist and antagonist groups for demographic and baseline data.

One hundred and four patients (90.4%) out of 115 patients received only one 3 mg dose of Cetrorelix. If the criteria for triggering of ovulation were not reached within four days (the protection period), we administered an additional dose of Cetrorelix (0.25 mg). Only nine (7.9%) of the patients received one additional dose on the morning of the hCG and two patients (1.7%) received two additional doses of 0.25 mg.

Moreover, a total of 18 patients of the Cetrorelix group (15.7%) presented an LH rise (LH > 10 IU/L) on the day of Cetrorelix injection. The administration of the Cetrorelix inhibited LH secretion. Four of them became pregnant (22.2%). These interrupted LH rises seem to have no measurable deleterious effect in this study. Only one patient in the Triptorelin group (2.8%) experienced an LH surge. None of the 115 patients of the Cetrorelix group experienced an LH surge after the Cetrorelix administration. No LH surge has been reported so far within the four days following the single administration of 3 mg Cetrorelix.

The mean length of stimulation was significantly lower in the Cetrorelix group. The mean number of ampoules was significantly higher in the

Triptorelin group. The E2 levels on the day of hCG were significantly lower in the Cetrorelix than in the Triptorelin group. The total number of follicles $\geq$15 and $\leq$17 mm was higher in the Triptorelin group (5.0 ± 3.9 vs. 3.4 ± 2.6; CI 0.5–2.8). The long GnRH agonist protocol resulted in more oocytes and more embryos as already demonstrated in the literature when compared to other stimulation regimens. However, the percentage of mature oocytes, fertilization rate, clinical and ongoing pregnancy rates, and miscarriage rates were not statistically different between the two groups. The incidence of ovarian hyperstimulation syndrome (OHSS) was lower in the GnRH-nt group. This difference did not reach statistical significance but some patients of the GnRH-a group were cancelled for being at risk of OHSS. Adding these patients brought the difference to significance.

In conclusion, this study has confirmed the efficacy of a single dose of 3 mg of Cetrorelix, administered in the late follicular phase, in preventing premature ovulation as indicated by LH surges. The single-dose protocol is easy to use and assures patient compliance. The 3 mg dose of Cetrorelix was tolerated well, with only mild and transitory reactions at the injection site. This protocol provides a shorter duration of treatment, uses less gonadotrophins, and has a lower incidence of OHSS. In some of the patients treated with rec-FSH, a decrease in the E2 level is observed after the injection of the Cetrorelix. This was also observed in our first study using a higher dose of Cetrorelix (5 mg) with human menopausal gonadotrophin (hMG) (11). An increase of the hMG dose, on the day of the antagonist administration, suppresses most of these E2 decreases, probably related to the LH suppression but not exclusively (19). However, no difference is observed in our experience in the IVF-ET results in the patients with or without an E2 decrease following the Cetrorelix administration (unpublished results). One study done in an oocyte donor model (20) found a lower implantation rate of embryos coming from oocytes collected in patients with an E2 drop as compared to patients with continuous rise of E2.

Multiple-Dose Protocol

In all the studies presented, the multiple-dose protocol uses 0.25 mg/day of Cetrorelix or Ganirelix. To compare the antagonist multiple dose protocol (0.25 mg/day) to the GnRH-a in IVF cycles, the European Cetrorelix Study Group (21) published the results of an open randomized trial. They studied 188 patients treated with Cetrorelix and 85 patients treated with the long (Buserilin) agonist protocol; both groups received hMG. The authors transferred embryos in 83.5% of Cetrorelix group versus 79% of Buserelin group. The clinical pregnancy rate was 22.3% and 25.9% per started cycle in the Cetrorelix and Buserilin groups, respectively; these differences were not statistically significant. The duration of treatment with gonadotropins and the estradiol serum levels on the day of hCG were lower in the antagonist

group. The incidence of ovarian hyperstimulation syndrome (OHSS II and III) was higher in patients using agonist treatment.

The European Ganirelix Study Group (22) also performed a controlled, multicentric, randomized trial to compare two treatment regimens for ovarian stimulation (multiple-dose antagonist vs. long-agonist) in women receiving recombinant FSH. A total of 672 patients were investigated and randomized. The total dose of FSH administered was higher in the Buserilin group (1500 and 1800 IU). In addition, patients receiving antagonist had a shorter stimulation duration than the agonist group. The estradiol serum levels on the day of hCG administration were higher in patients using Buserelin than Ganirelix and the incidence of OHSS was higher (5.9 vs. 2.4%) in the Buserelin group. Otherwise, the number of good quality embryos, fertilization rate (62.1% in both groups), and replaced embryos were similar between the two treatments schemes. The implantation rate was lower in the Ganirelix group (15.7%) than in Buserelin group (21.8%), however the clinical pregnancy rates per attempt were not statistically significant.

The North American Ganirelix Study Group (23) was organized to evaluate the efficacy and safety of Ganirelix (multiple-dose protocol) versus leuprolide (long-protocol) in IVF patients. This multicenter (United States and Canada) trial demonstrated that the mean number of retrieved oocytes was similar between the groups (11.6 in antagonist group and 14.1 in agonist group). Moreover, the fertilization rates (62.4% and 61.9%) and implantation rates (21.6% and 26.1%) were also similar in both groups. The ongoing pregnancy rates per attempt were 30.8% in ganirelix group and 36.4% in leuprolide group; however, the antagonist group showed fewer local site reactions after injection administration (12.5%) than the leuprolide group (25.5%). The authors proved the effectiveness and safety of multiple antagonist drug protocol with a shorter stimulation period and fewer side effects when compared with the long agonist (leuprolide) protocol.

Another multicentric European (The European and Middle East) Orgalutran Study Group (24) trial, comparing two treatment schemes (Ganirelix and Triptorelin) in 337 women, demonstrated that the median dose of FSH recombinant was lower in the antagonist protocol. The authors showed also that the estradiol serum levels were lower in Ganirelix group on the day of hCG. The fertilization rates (64% Ganirelix and 64.9% Triptorelin), the mean number of good quality embryos (2.7 and 2.9, respectively), the implantation rates (22.9% both treatments), and finally the ongoing pregnancy rate per attempt were similar between the two treatments (31% and 33.9%, Ganirelix and Triptorelin, respectively).

The multiple-dose protocol, compared with the long-agonist regimen, offers a simple, safe, and efficient option, with comparable IVF results. The OHSS risk is decreased (25), the total dose of gonadotrophin needed to stimulate the ovulation is lower, and the stimulation period is also shorter than in the long protocol. Patients receiving antagonist treatment had lower

estradiol serum levels at the time of hCG administration, probably because of the lower number of follicles. The impact of this finding in implantation rates is disputed and unknown.

In the multiple-dose protocol, there is a small incidence of LH surge (between 1% and 2.5%). These surges were often associated with a lack of compliance by the patients, forgetting one antagonist administration. This point is important to stress to the patients. More recently, some centers have observed a higher incidence of LH surge in poor responders using the multiple-dose protocol (unpublished data). These reports should be confirmed and documented. The dose of 0.25 mg might not be always sufficient. This dose might also have to be adapted to the weight of the patients.

The follicular development was also studied in a controlled randomized multicentric study, in patients using Ganirelix with different doses (0.0625–2.0 mg/day). Patients received recombinant FSH after day 2 of menstrual cycle and Ganirelix were administrated daily after day 6 of ovarian stimulation protocol (26). Overall, 311 patients were studied and compared in terms of number of follicles, total follicular surface area, serum gonadotropin, and steroid hormones levels. Increasing GnRH-nt doses demonstrated an additional suppressive action on estradiol and androstenedione serum levels, probably by an important inhibition of LH secretion, which may have exerted a harmful effect. The follicular growing pattern was not affected by the dose of GnRH-nt. The decreased secretion of androstenedione and estradiol was not totally explained by the LH inhibition. Other mechanisms could be involved in GnRH-nt action and influence the cycles stimulated with this regimen protocol.

Wikland et al. (27), in a prospective randomized trial, evaluate two starting doses (150 vs. 225 IU) of recombinant FSH with the multiple-dose Cetrorelix protocol. The purpose was to increase the number of follicles, oocytes, and embryos to increase the pregnancy rates. Despite a higher number of recovered oocytes in the group of patients receiving 225 IU of recombinant FSH, pregnancy and implantation rates were similar.

The effect of GnRH-nt on oocyte and embryo quality could also be measured by studying the implantation and pregnancy rates after cryopreservation of pronuclear oocytes or embryos. The first study to evaluate this aspect was conducted with 62 patients divided into two groups (28). One group received the multiple GnRH-nt dose protocol (group I) and the other group the conventional GnRH long protocol (group II). The implantation and pregnancy rates, after frozen-thawed procedure in pronucleous oocyte stage, were similar between the groups (3.26% and 8.33% for group I; 3.73% and 10.25% for the group II).

GnRH-nt IN MILD STIMULATION

Mild stimulation protocol is aimed at reducing the intensity of stimulation to obtain a lower number of oocytes. The advantages of those regimens

are a reduction in the intensity of side effects, a reduction in the incidence of OHSS, and a reduction in costs and complication. Other advantages lie in the potential adverse effects on endometrium of heavy stimulation regimens and the possible benefit of a natural selection of the best oocytes.

Those protocols include mainly the association between citrate of clomiphene and gonadotrophins, the modified natural cycle, and the reduced amount of gonadotrophin dose. In both these protocols, the GnRH-nts were used to prevent LH surges.

Very few data are available on the use of CC-hMG/rec-FSH and antagonists in IVF (29,30). Pregnancy rates appear satisfactory but a high incidence of LH surges was observed (31). It was demonstrated in an animal model that CC increases the sensitivity of the pituitary to GnRH and that the dose of antagonists might need to be increased when CC is used. A prospective study found a lower pregnancy rate in CC-hMG cycles and did not support the interest in this protocol (32).

The revival of the natural cycle was proposed as the use of GnRH-nts could prevent premature LH surges. The objective of this regimen is to combine the possible prevention of an LH surge by the administration of the GnRH-nt and the simplicity of the natural cycle with minimal stimulation (Fig. 2). We investigated the administration of the Cetrorelix in the late follicular phase of minimally stimulated cycles in women of good prognosis. These patients had an age between 26 and 36 years old (mean 34.1 ± 1.4), normal menstrual cycles, day 3 FSH < 8 UI/l, day 3 E2 < 50 pg/ml, less than three previous IVF procedures, male factor infertility requiring IVF, and ICSI (33). A single subcutaneous injection of 1 or 0.5 mg Cetrorelix was administered when plasma estradiol levels reached 100–150 pg/ml, and a lead follicle was between 12 and 14 mm to assess the minimal effective dose. Since studies with Nal–Glu (34) and Cetrorelix (35) demonstrated that estradiol secretion can be reduced after the GnRH-nt administration, daily administrations of 150 IU of hMG were performed at the time of the first injection of Cetrorelix and repeated thereafter until hCG administration.

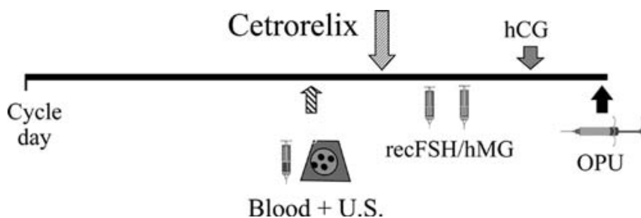

Figure 2 Modified natural cycle using Gonadotropin-releasing hormone antagonists. *Abbreviations*: hCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; hMG, human menopausal gonadotropin; OPU, oocyte pick-up.

This treatment scheme is not a complete natural cycle since a low gonadotrophin support is associated (minimal stimulation). Triggering of ovulation (5000 IU of hCG) was decided when the lead follicle reached 16–20 mm and estradiol values were above 200 pg/ml. Oocyte pick-up was performed 36 to 40 hours later without anesthesia (36).

A total of 33 patients (44 cycles) were included. The mean number of hMG ampoules was 4.7 ± 1.4 and the mean time between the Cetrorelix and hCG administration was 2.0 ± 0.7 days. We canceled four cycles (9.0%). Follicular growth and E2 secretion were not altered by the Cetrorelix administration. A total of 40 oocytes retrievals leading to 22 transfers (55%) were performed. In 10 cycles, no oocyte was obtained. Fertilization failure occurred in six cycles, and in two patients the transfer was not performed because of a developmental arrest of the embryo at the two pronuclear stages. The fertilization rate was 80% (24 embryos/30 oocytes). A total of five clinical pregnancies were obtained (32.0%/transfer, 17.5%/retrieval) of which four are ongoing.

The number of patients in whom the cycle was canceled for premature LH surge was very low (9.0%) as compared to previous reports on natural cycles, confirming the efficacy of the antagonist administration. In addition, the pregnancy rate (17.5% clinical pregnancy rate per retrieval; 32.0% per transfer) seems interesting, even though it has to be confirmed in larger series. The same protocol was used in a recent study with rec-FSH as follicular growth support (37).

Another form of mild stimulation was proposed recently by delaying the start of gonadotrophin stimulation. The mild ovarian stimulation protocol resulted in pregnancy rates per started IVF cycle similar to those observed after profound stimulation with GnRH agonist cotreatment, despite shorter stimulation and a 27% reduction in exogenous FSH. A higher cancellation rate before oocyte retrieval was compensated by improved embryo quality concomitant with a higher chance of undergoing ET (38).

The high burden and drawbacks of "heavy" (stimulated) COH protocol (side effects, multiple pregnancies, and potential serious health complications) make a clear demand for softer protocols (39,40), a "friendly IVF" (41).

If these preliminary results with spontaneous cycle and hMG support are confirmed on large numbers, the repetition of two or three of these cycles could lead to acceptable cumulative pregnancy rates without the potential adverse effects of COH (42–44) and be more cost-effective (45).

Another use of natural cycle with GnRH-nt was proposed recently. The protocol was proposed in poor responders (46). The number of studies evaluating this approach is very low so far and results are controversial (47). Further studies are needed to analyze the possible interest of the modified natural cycle in poor responders.

REMAINING QUESTIONS ON GnRH-nt

Pregnancy Rates

There is a trend in most of the controlled studies using GnRH-nt (with both compounds and protocols) to find slightly lower pregnancy rates as compared to the GnRH-a long protocol. This led to the questioning of IVF-ET results of GnRH-nts. A meta-analysis concluded there were significantly lower pregnancy rates in GnRH-nt cycles as compared to GnRH agonists (48). However, the difference was very close to being nonsignificant (OR 0.79; 95% CI 0.63–0.99). Adding one study, Ludwig et al. (49) did not find a significant difference in pregnancy rates for Cetrorelix. A recent meta-analysis presented by Daya did not find a difference in ongoing pregnancy rate (unpublished data). Care should be taken in drawing conclusions on these observations. Some population factors were not equivalent in the groups despite randomization. In addition, the learning curve, inherent to the use of new treatment schemes, could have influenced some of the studied outcome. The trend towards higher pregnancy rate (PR) in the GnRH-a group may be associated with the relatively higher number of obtained embryos due to the higher number of oocytes. This hypothesis was not confirmed by the study presented by Wikland et al. (27). The difference could be related to the absence of desensitization of the previous luteal phase. In fact, a difference of the same magnitude in the pregnancy rates is found between the short and long protocols (50). The clear reasons for this difference are still not clear. The potential deleterious effect of the GnRH-nt on the endometrium or even on the fertilization process has been presented (51). However, there is no clinical data to confirm these hypotheses in the human species (52,53). Therefore, a careful analysis is needed before drawing conclusions on the PR. In fact, a comparative study designed to assess a 5% difference for PR situated in the region of 20% will require over 1200 patients in each treatment group.

The important issue about GnRH-nt pregnancy rates is that there are still major questions to be answered on the best way to use them.

Single Versus Multiple-Dose Protocols

The two protocols have not been prospectively compared in large studies. The only large available study presents the results of two large multicentric studies using the single- and multiple-dose Cetrorelix protocols (54). No differences are observed between the two regimens but as this is not a prospective randomized study; firm conclusions cannot be drawn from those data. A propspective randomized study compared the Ganirelix multiple dose protocol to the Cetroreliw single dose regimen and did not find a statistical difference in the PR between the two protocols (55,56).

Fixed Versus Flexible Administration of the Antagonist

In the first study using GnRH-nt, the protocol included a fixed day of GnRH-nt administration. In the multiple-dose protocol, the antagonist

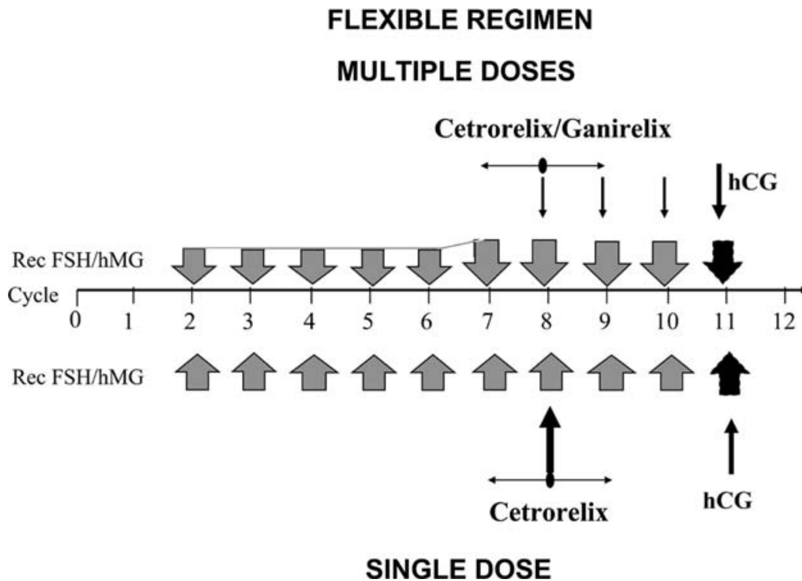

Figure 3 Gonadotropin-releasing hormone antagonist multiple- and single-dose protocols. Fixed day regimens. *Abbreviations*: FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin.

was started on day 6. In the single dose, the 3 mg administration of Cetrorelix was proposed on day 7. We proposed a more flexible approach by adapting the moment of antagonist administration to ovarian response (Fig. 3) (11). The antagonist could be administered when the leading follicle reached 14 mm and/or E2 reached 600 pg/ml. The same approach was later proposed by Ludwig et al. (57) in the multiple-dose protocol (Fig. 3). Some authors have predicted a lower pregnancy rate when the flexible approach was proposed (58). Data remain controversial on this question. A recent meta-analysis did not find differences in the pregnancy rates between the flexible and fixed approaches (59).

A very early start of the antagonist, injected at the beginning of the stimulation, was proposed to reduce the LH levels at the beginning of the follicular phase. A prospective randomized study failed to demonstrate an advantage to this proposal (60). Moreover, a long period of injection of antagonist will really alter the main benefit of GnRH-nts.

INDICATIONS

PCOS Patients

The use of GnRH-nts in large series of polycystic ovarian syndrome (PCOS) patients has not been published so far. One of the most important hormonal

aspects of PCOS patients is the increased LH tone secretion. This group of patients is characterized by anovulation, and ovarian ovulation induction is usually performed using clomiphene citrate, FSH associated or not with GnRH agonists. The rationale for the use of GnRH-nt in PCOS patients is the fact that the LH/FSH ratio will be decreased since LH secretion is more affected by the antagonist administration than FSH secretion (2). In IVF, another clear advantage is the reduced incidence of OHSS with the utilization of GnRH-nt. The use of GnRH-nt protocol allows also to induce the oocyte final maturation with GnRH agonist, to elicit an endogenous LH surge and, subsequently, decreasing the risk of OHSS (61). However, a large prospective trial is necessary to confirm these physiological hypotheses (see infra). Lubin et al. (62) described two case reports of PCOS patients treated with GnRH-nt before the treatment with GnRH agonist to induce ovulation. The patients showed a normalization of LH and testosterone serum levels, however, the authors failed to induce an appropriate ovarian response.

Two recent prospective randomized studies compared GnRH-nt to agonists in PCO patients (63,64). Those studies found similar pregnancy rate but failed to find a significant advantage to GnRH-nts.

Larger studies are needed to further evaluate the potential benefits of the association of GnRH-nt in PCOS patients.

POOR RESPONDERS

The definition of poor responders and the heterogeneity of this group of patients cause an important bias in published series. The rational for using ovarian stimulation protocols with GnRH-nt in poor responders is that GnRH-nts do not require desensitization and are not causing an important depression on gonadotropin secretion during the stimulation.

Forty-two patients who are poor responders were divided into two groups for ICSI treatment (long GnRH agonist or Cetrorelix multiple-dose protocols) (65). The stimulation protocol also included, in some patients, clomiphene citrate associated with gonadotropins. Age, number of oocytes retrieved, number of fertilized oocytes, transferred embryos, score of embryo quality, and clinical pregnancy were not significantly different between the groups. A trend was observed in the pregnancy rates (14.28% for Cetrorelix vs. 9.52% for GnRH agonist treatment) but the difference was not significant. The authors discussed the sample size utilized. Probably, with an adequate power calculation this difference in terms of pregnancy rate can become statistically important.

With the same objective of the above-mentioned paper, Akman et al. (66) presented a randomized trial comparing the microdose flare-up GnRH-a protocol versus the antagonist multiple dose protocol. Forty-eight patients were divided into the two regimen protocols. The implantation rates (15.07% for flare-up protocol and 11.36% for Cetrorelix) and the ongoing pregnancy

rates per transfer (21.05% and 16.6% for flare-up and Cetrorelix protocol, respectively) were similar between the two groups.

Other small studies have been published recently (67–69). Those studies did not find an improvement in pregnancy rates, but similar results were achieved.

The Need for LH Activity Adjunction

The need to counteract the dramatic decrease in LH observed in the GnRH-nts cycle has been proposed. No large prospective study is yet published comparing patients treated with GnRH-nt in which LH activity was added. LH activity could be added by the adjunction of rec-LH or by a small dose of hCG (70). It could also be obtained by using hMG. Profound suppression of LH was not found to be associated with lower pregnancy rates (71). No significant advantage was found by adding rec-LH on a systematic basis in GnRH-nts single and multiple-dose protocols (72,73). This remains to be confirmed in large published studies.

Programmation of GnRH-nt Cycles

The programmation of GnRH-nt cycles is important to organize the work load of large IVF centers. Programmation could be obtained with progesterone administration in the late luteal phase or oral contraceptive pill (74). A more original way of programming the cycle was proposed by Fanchin et al. (75). The authors proposed luteal administration of estradiol in the late luteal phase as proposed by de Ziegler (76). The administration of estradiol prevents the FSH rise induced by the luteolysis and could synchronize the follicular cohort (75). Some authors (Kolibianakis et al., unpublished data) found lower pregnancy rates when an estro-progestative pill is used. The results of this study are not yet confirmed by other authors. In poor responders, the use of contraceptive pills to program GnRH-nt cycle was found to be associated with increased cancellation rate (77).

Luteal Phase

The LH secretion is fundamental for the development of a normal luteal phase and for progesterone secretion. The luteal-phase defect induced by agonist administration is well known and studied, caused mainly by the profound pituitary suppression (78). The antagonist exerts a transitory LH inhibition and, hypothetically, the luteal phase is less disturbed. Some authors (78,79), comparing LH serum levels in the early and mid-luteal phase of hMG treated cycles with or without antagonist (Cetrorelix multiple dose), concluded that there is a decrease in LH serum levels in Cetrorelix groups. However, the implications of this phenomenon were not studied. In a small group of patients treated with Cetrorelix multiple doses without

luteal support, no pregnancy was obtained (80). A recent study comparing triggering of ovulation with hCG, rec-LH, and Triptoreline in IVF patients without any luteal support and treated with GnRH-nts showed an abnormal luteal phase in those three regimens (81). However, it must be emphasized that the authors used Antide as an antagonist and those results might not be extrapolated to Cetrorelix or Ganirelix. Others (82) demonstrated that, comparing antagonist and agonist treatment, the granulosa cells cultured in vitro from IVF patients were less impaired, in terms of progesterone secretion, in the antagonist group. Ragni et al. (83) showed, on intrauterine insemination (IUI) cycles, that the utilization of GnRH-nt is safe and do not affect the luteal-phase duration or progesterone secretion. The fact that the intensity of the stimulation is much lower in IIU cycles could explain those contradictory results, as luteal phase is affected by strong stimulation regimens.

Until full scientific data and controlled studies are available, it seems preferable to maintain luteal support of GnRH-nt treated cycles.

Triggering Ovulation with GnRH-a in GnRH-nts Cycles

We described the use of GnRH agonist to induce endogenous LH surge during an ovarian stimulation cycle with GnRH-nt (61). All patients showed an appropriate LH and progesterone rise after the GnRH agonist administration, confirming that this approach can be used to induce LH secretion during the final stage of COH.

Others (84–87) previously proposed this strategy to decrease the risk of OHSS, as the endogenous LH has a lower half-life than hCG. However, this approach is not suitable in patients previously down-regulated with GnRH agonist.

A recent study, comparing hCG, Lupron (0.2 mg), and Triptorelin (0.1 mg) to trigger ovulation in IVF patients treated with Ganirelix, found similar IVF-ET results between the three groups of patients (88). Other studies found lower pregnancy rates in patients treated with a combination of GnRH-nts and agonists (89). A small group of high responders were treated with a combination of GnRH-nt and agonists and no OHSS was observed in this preliminary report (90). As mentioned earlier, a study using Antide as GnRH-nt showed an altered luteal phase in cycles combining GnRH-nt and agonist (81). Some authors advocate that the luteal phase needs to be supported by both progesterone and estrogens (Itskowitz, personal communication). In a prospective randomized study, reduced pregnancy rate was found in GnRH-nt cycle in which GnRH-a was used to trigger ovulation, despite using progesterone and estradiol as luteal supplementation (91).

More studies are needed before recommending this approach.

IUI

Few papers on the use and potential benefits of GnRH-nt protocol in IUI cycles are yet available. Some found higher pregnancy rates (92,93). Others found only a trend in higher success rate (94).

Some of the advantages shown in IVF cycles can be applied for IUI. In case of premature LH surge when criteria of optimal follicular maturation are not obtained, GnRH-nts could be proposed to prevent and postpone ovulation. The luteal phase of stimulated cycle in IUI cycles was studied by Ragni et al. (83).

Programmation of the timing of IUI could also be obtained with GnRH-nt. Of course, this is not a medical indication and IUI can be advanced if an LH surge is detected, however this is not always possible. It remains to be demonstrated that postponing the triggering of ovulation with GnRH-nt when adequate follicular size and E2 levels are reached does not adversely affect the results.

Perinatal Outcome of Pregnancy After GnRH-nt for Ovarian Induction

Recently, two papers have been published on the perinatal outcome of IVF pregnancies obtained with GnRH-nt.

One report followed 67 pregnant patients after ovarian induction with Ganirelix multiple-dose protocol (95). The miscarriage rate was 9%, and full data on perinatal outcome was obtained in 61 patients. The mean gestational age was 39.4 week for singleton pregnancies and 36.6 week for multiple pregnancies. A birth weight lower than 2500 g was present in 8.7%, one baby had a major congenital malformation, and seven minor malformations were reported in five infants. These results were not different from data available on IVF pregnancies.

Another study addresses the same objective with the use of Cetrorelix (multiple- and single-dose protocols). Pregnancies that resulted from phase II and III trials were followed to investigate the safety of GnRH-nt (96). A total of 227 children born were evaluated in terms of outcome of pregnancy, delivery, birth weight, and after one and two years of age to search for some developmental disorder. The incidence of major congenital malformation was 3.1% and minor malformations occurred in 2.6% of the cases. The clinical abortion rate was 16.8% and the ectopic pregnancy rate 3.4%. The follow-up data on physical development did not show any significantly abnormality.

The authors of both studies concluded that the use of GnRH-nt in ovarian stimulation protocols did not cause a harm or detrimental effect on the pregnancy course or perinatal outcome of those patients. These two studies concern a too small number of cases to discuss the malformations rates.

Recommended Regimens

As we saw in this paper we still have questions on the best protocol to be used with GnRH-nts. From available literature, we can suggest that GnRH-nt can be used in a single- or multiple-dose regimen. The flexible or fixed approach appears also to give similar results. Programming the cycle can be obtained with OC pill or luteal estrogen. The latter lacks a prospective study to confirm its interest as opposite to the OC pill which is already used in many IVF cycles. The supplementation with LH activity is not supported by available scientific data. The supplementation of the luteal phase is recommended. If GnRH-nt appears to give comparable results in good responders, it should be studied in poor responders, patients with PCOS or IUI deserve further study.

CONCLUSION

In COH, the different studies presented have confirmed the efficacy of a single dose of 3 mg of Cetrorelix to prevent premature LH surges when administered in the late follicular phase. The single-dose protocol is easy to use and assures patient compliance. When compared with the long protocol using a depot formula of Triptorelin, the IVF-ET results showed a shorter duration of treatment, less amount of hMG used, and a lower occurrence of OHSS in the group of patients treated with Cetrorelix. Clinical trials showed that the multiple-dose protocol using Cetrorelix or Ganirelix is effective and safe. A shorter duration of treatment, less amount of gonadotropins, and a lower occurrence of OHSS was observed in the group of patients treated with Cetrorelix or Ganirelix. Single- and multiple-dose protocols have not yet been compared prospectively. Fixed day or flexible day schemes have not been compared either. The single dose is simple but requires monitoring of the cycle. The multiple doses, in a fixed regimen, could reduce the need for hormone assessments but compliance is mandatory. Patients report a better quality of life with GnRH-nt protocol as compared to the long GnRH-a regimen, but this aspect was not scientifically evaluated.

The pregnancy rates are not statistically different within the GnRH agonist treatment. A trend in lower pregnancy rates observed in most of the antagonists group of the controlled studies suggests further data are needed on this point. Meta-analyses found the difference significant in favor of the GnRH agonist.

However, there are still major questions to be answered to define the best way to use GnRH-nts (97). Therefore, it should be mentioned that there is room for optimization of the antagonist protocol. The antagonist administration can be proposed on a fixed day of the stimulation or based on monitoring with a flexible approach. This could reduce the amount of

antagonists. However, the optimal timing of the flexible approach of GnRH-nt administration is difficult since prediction of LH surge is difficult. The programmation of the treatment through manipulation of the luteal phase with progestative and/or estrogen has to be evaluated. The luteal-phase supplementation remains mandatory so far.

The use of the GnRH-nt in mild stimulation regimen (CC/gonadotropins or natural cycle with hMG support) allows reduction of the rate of premature LH surges and therefore the cancellation rate. Stimulation can be minimal and pregnancy rates, in some preliminary report, are satisfactory. If larger studies confirm these results, mild stimulation protocols associated with GnRH-nt single or multiple dose administration could represent an interesting first-choice IVF treatment regimen in selected indications. These protocols could reduce the complications and risks of the actual COH protocols. The reduction of the costs associated with the possibility of offering the retrieval on an outpatient procedure is also of clear interest. Successive cycles with an acceptable success rate could result in interesting cumulative pregnancy rates. More studies are needed to clarify some important clinical questions regarding the utilization of antagonist in PCO patients and in poor responders. In IUI cycles, small studies showed an increase in pregnancy rate when GnRH-nt multiple-dose regimens were use in the stimulation regimen. These results need to be confirmed on larger samples.

New GnRH-nts are already available for clinical use in most countries. It will certainly change our protocols of ovarian stimulation. If similar pregnancy rates are achieved by a better understanding of the effect of GnRH-nts, the main advantage of these compounds is the reduction of the side effects and complications of our actual stimulation protocols, a clear benefit for our patients. They could also allow us to design softer stimulation schemes.

REFERENCES

1. Karten MJ, Hoeger CA, Hooh WA. The development of safer antagonists: strategy and status. In: Bouchard P, Haour F, Franchimont P, Schatz B, eds. Recent Progress on LH–RH and Gonadal Peplides. Paris: Elsevier, 1990:147–158.
2. Reissmann T, Felberbaum R, Diedrich K, Engel J, Comaru-Schally AM, Schally AV. Development and applications of luteinizing hormone-releasing antagonists in the treatment of infertility: an overview. Hum Reprod 1995; 10:1974–1981.
3. Matikainen T, Ding YQ, Vergara M, Huhtaniemi I, Couzinet, B, Schaison G. Differing responses of plasma bioactive and immunoreactive follicle-stimulating hormone and luteinizing hormone to gonadotropin-releasing hormone antagonist and agonist treatments in postmenopausal women. J Clin Endocrinol Metab 1992; 75:820–825.
4. Bouchard P, Charbonnel B, Caraty A, et al. The role of LHRH during the periovulatory period: a basis for the use of LHRH antagonists in ovulation induction. In: Filicori M, Flamigni C, eds. Ovulation: Basic Science and Clinical Advances. Elsevier Science BV International Congress Series 1046, 1994.

5. Dubourdieu S, Charbonnel B, d'Acremont MF, Carreau S, Spitz IM, Bouchard P. Effect of a GNRH antagonist (Nal–Glu) during the periovulatory period: the LH surge requires secretion of gonadotropin-releasing hormone. J Clin Endocrin Metab 1994; 78:343–347.
6. Olivennes F, Belaisch-Allart J, Emperaire JC, et al. A prospective randomized controlled study in IVF-ET with a single dose of a LH–RH antagonist (Cetrorelix) or a depot formula of a LH–RH agonist (Triptorelin). Fertil Steril 2000; 73: 314–320.
7. Diedrich K, Diedrich E, Santos E, et al. Suppression of the endogenous LH-surge by the GNRH antagonist Cetrorelix during ovarian stimulation. Hum Reprod 1994; 9:788–791.
8. Albano C, Smitz J, Camus M, Riethmuller-Winzen H, Van Steirteghem A, Devroey P. Comparison of different doses of gonadotropin-releasing hormone antagonist Cetrorelix during controlled ovarian hyperstimulation. Fertil Steril 1997; 67:917–922.
9. The Ganirelix Dose-finding Study Group. A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (puregon). Hum Reprod 1998; 13:3023–3031.
10. Olivennes F, Fanchin R, Bouchard P, et al. The single or dual administration of the gonadotrophin-releasing hormone antagonist Cetrorelix in an in vitro fertilization embryo transfer program. Fertil Steril 1994; 62:468–476.
11. Olivennes F, Fanchin R, Bouchard P, et al. Scheduled administration of GNRH antagonist (Cetrorelix) on day 8 of in vitro fertilization cycles: a pilot study. Hum Reprod 1995; 10:1382–1386.
12. Frydman R, Cornel C, de Ziegler D, Taieb J, Spitz IM, Bouchard P. Prevention of premature luteinizing hormone and progesterone rise with a GnRH antagonist Nal–Glu in controlled ovarian hyperstimulation. Fertil Steril 1991; 56:923–927.
13. Frydman R, Cornel C, de Ziegler D, Taieb J, Spitz IM, Bouchard P. Spontaneous luteinizing hormone surges can be reliably prevented by the timely administration of a gonadotrophin releasing hormone antagonist (Nal–Glu) during the late follicular phase. Hum Reprod 1992; 7:930–933.
14. Olivennes F, Alvarez S, Bouchard P, Fanchin R, Salat-Baroux R, Frydman R. The use of a new GnRH antagonist (Cetrorelix) in IVF-ET with a single dose protocol: a dose finding study of 3 versus 2 mg. Hum Reprod 1998; 13: 2411–2414.
15. Christin-Maitre S, Olivennes F, Dubourdieu S, et al. Effect of GnRH antagonist during the LH surge in normal women and during controlled ovarian hyperstimulation. Clinic Endocrinol 2000; 52:721–726.
16. Sommer L, Zanger K, Dyong T, et al. Seven-day administration of gonadotrophin releasing hormone antagonist Cetrorelix in normal cycling women. Eur J Endocrinol 1994; 13:280–285.
17. Felberbaum R, Reissmann T, Kupker W, et al. Hormone profiles under ovarian stimulation with human menopausal gonadotrophin (HMG) and concomitant administration of the gonadotropin releasing hormone GnRH—antagonist Cetrorelix at different dosages. J Assist Reprod Genet 1996; 13:216–222.

18. Kol S, Lightman A, Hillensjo T, et al. High doses of gonadotrophin-releasing hormone antagonist in in vitro fertilization cycles do not adversely affect the outcome of subsequent freeze-thaw cycles. Hum Reprod 1999; 14:2242–2244.
19. de Jong D, Macklon NS, Eijkemans MJ, Mannaerts BM, Coelingh Bennink HJ, Fauser BC. Dynamics of the development of multiple follicles during ovarian stimulation for in vitro fertilization using recombinant follicle-stimulating hormone (puregon) and various doses of the gonadotropin-releasing hormone antagonist ganirelix (Orgalutran/Antagon). Fertil Steril 2001; 75:688–693.
20. Lindheim SR, Morales AJ. GnRH antagonists followed by a decline in serum estradiol results in adverse outcomes in donor oocyte cycles. Hum Reprod 2003; 18:2048–2051.
21. Albano C, Felberbaum RE, Smitz J, et al. European Cetrorelix Study Group. Ovarian stimulation with HMG: results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist Cetrorelix and the LHRH-agonist Buserelin. Hum Reprod 2000; 15:526–531.
22. Borm G, Mannaerts B. The European Orgalutran Study Group. Treatment with the gonadotrophin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe and convenient: results of a controlled, randomized, multicentre trial. Hum Reprod 2000; 15:1490–1498.
23. The North American Ganirelix Study Group. Efficacy and safety of ganirelix acetate versus leuprolide acetate in women undergoing controlled ovarian hyperstimulation. Fertil Steril 2001; 75:38–45.
24. The European and Middle East Orgalutran Study Group. Comparable clinical outcome using the GnRH antagonist ganirelix or a long protocol of the GnRH agonist Triptorelin for the prevention of premature LH surges in women undergoing ovarian stimulation. Hum Reprod 2001; 16:644–651.
25. Ludwig M, Felberbaum RE, Devroey P, et al. Significant reduction of the incidence of ovarian hyperstimulation syndrome (OHSS) by using the LHRH antagonist Cetrorelix (Cetrotide) in controlled ovarian stimulation for assisted reproduction. Arch Gynecol Obstet 2000; 264:29–32.
26. De Jong D, Van Hooren EG, Macklon NS, Mannaerts BM, Fauser BC. Pregnancy and birth after GnRH agonist treatment for induction of final oocyte maturation in a woman undergoing ovarian stimulation for ICSI, using a GnRH antagonist (Orgalutran/Antagon) to prevent a premature LH surge: a case report. J Assist Reprod Genet 2001; 18:30–33.
27. Wikland M, Bergh C, Borg K, et al. A prospective, randomized comparison of two starting doses of recombinant FSH in combination with Cetrorelix in women undergoing ovarian stimulation for IVF/ICSI. Hum Reprod 2001; 16:1676–1681.
28. Nikolettos N, Al-Hasani S, Felberbaum R, et al. Comparison of cryopreservation outcome with human pronuclear stage oocytes obtained by the GnRH antagonist, Cetrorelix, and GnRH agonist. Eur J Obstet Gynecol Reprod Biol 2000; 93:91–95.
29. Engel JB, Ludwig M, Felberbaum R, Albano C, Devroey P, Diedrich K. Use of Cetrorelix in combination with clomiphene citrate and gonadotrophins: a suitable approach to 'friendly IVF'? Hum Reprod 2002; 17:2022–2026.

30. Engel JB, Olivennes F, Fanchin R, et al. Single dose application of Cetrorelix in combination with clomiphene for friendly IVF: results of a feasibility study. Reprod Biomed Online 2003; 6:444–447.
31. Engel JB, Felberbaum RE, Eilers W, Polack S, Ortmann O, Diedrich K. Clomiphene-induced LH surges and Cetrorelix. Reprod Biomed Online 2002; 5: 109–111.
32. Mansour R, Aboulghar M, Serour GI, Al-Inany HG, Fahmy I, Amin Y. The use of clomiphene citrate/human menopausal gonadotrophins in conjunction with GnRH antagonist in an IVF/ICSI program is not a cost effective protocol. Acta Obstet Gynecol Scand 2003; 82:48–52.
33. Rongières-Bertrand C, Olivennes F, Righini C, et al. Revival of the natural cycles in in vitro fertilization with the use of a new gonadotropin-releasing hormone antagonist (Cetrorelix): a pilot study with minimal stimulation. Hum Reprod 1999; 14:683–688.
34. Kettel LM, Roseff SJ, Chiu TC, et al. Follicular arrest during the midfollicular phase of the menstrual cycle: a gonadotropin-releasing hormone antagonist imposed follicular–follicular transition. J Clin Endocrinol Metab 1991; 73:644–649.
35. Leroy I, d'Acremont MF, Brailly-Tabard S, Frydman R, de Mouzon J, Bouchard P. A single injection of gonadotropin-releasing hormone (GnRH) antagonist (Cetrorelix) postpones the luteinizing hormone (LH) surge: further evidence for the role of GnRH during the LH surge. Fertil Steril 1994; 62:461–467.
36. Ramsewak SS, Kumar A, Welsby R, Mowforth A, Lenton EA, Cooke ID. Is analgesia required for transvaginal single follicle aspiration in in vitro fertilization? a double blind study. J In Vitro Fertil Embr Transf 1990; 7:103–106.
37. Pelinck MJ, Vogel NE, Hoek A, Arts EG, Simons AH, Heineman MJ. Minimal stimulation IVF with late follicular phase administration of the GnRH antagonist Cetrorelix and concomitant substitution with recombinant FSH: a pilot study. Hum Reprod 2005; 20:642–648.
38. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. J Clin Endocrinol Metab 2003; 88:166–173.
39. Edwards R, Lobo R, Bouchard P. Time to revolutionize ovarian stimulation. Hum Reprod 1996; 11:917–919.
40. Fauser BC, Devroey P, Yen SS, et al. Minimal ovarian stimulation for IVF: appraisal of potential benefits and drawbacks. Hum Reprod 1999; 14:2681–2686.
41. Olivennes F, Frydman R. "Friendly IVF" the way of the future? Hum Reprod 1998; 13:1121–1124.
42. Lenton EA, Cooke ID, Hooper M, et al. In vitro fertilization in the natural cycle. Baillière Clin Obstet Gynaecol 1992; 6:229–244.
43. Ingerslev HJ, Hojgaard A, Hindkjaer J, Kesmodel U. A randomized study comparing IVF in the unstimulated cycle with IVF following clomiphene citrate. Hum Reprod 2001; 16:696–702.
44. Nargund G, Waterstone J, Bland J, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. Hum Reprod 2001; 16:259–262.

45. Daya S, Gunby J, Hughes EG, Collins JA, Sagle MA, YoungLai EV. Natural cycles for in-vitro fertilization: cost-effectiveness analysis and factors influencing outcome. Hum Reprod 1995; 10:1719–1724.
46. Ubaldi FM, Rienzi L, Ferrero S, et al. Management of poor responders in IVF. Reprod Biomed Online 2005; 10:235–246.
47. Kolibianakis E, Zikopoulos K, Camus M, Tournaye H, Van Steirteghem A, Devroey P. Modified natural cycle for IVF does not offer a realistic chance of parenthood in poor responders with high day 3 FSH levels, as a last resort prior to oocyte donation. Hum Reprod 2004; 19:2545–2549.
48. Al-Inany H, Aboulghar M. GnRH antagonist in assisted reproduction: a Cochrane review. Hum Reprod 2002; 17:874–885.
49. Ludwig M, Katalinic A, Diedrich K. Use of GnRH antagonists in ovarian stimulation for assisted reproductive technologies compared to the long protocol. Meta-analysis. Arch Gynecol Obstet 2001; 265:175–182.
50. Hughes EG, Fedorkow DM, Daya S, Sagle MA, Van de Koppel P, Collins JA. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: a meta-analysis of randomized controlled trials. Fertil Steril 1992; 58:888–896.
51. Hernandez ER. Embryo implantation and GnRH antagonists: embryo implantation: the Rubicon for GnRH antagonists. Hum Reprod 2000; 15:1211–1216.
52. Mannaerts B, Gordon K. Embryo implantation and GnRH antagonists: GnRH antagonists do not activate the GnRH receptor. Hum Reprod 2000; 15: 1882–1883.
53. Ortmann O, Weiss JM, Diedrich K. Embryo implantation and GnRH antagonists: ovarian actions of GnRH antagonists. Hum Reprod 2001; 16:608–611.
54. Olivennes F, Diedrich K, Frydman R, Felberbaum RE, Howles CM. Cetrotide Multiple Dose International Study Group, Cetrotide Single Dose International Study Group. Safety and efficacy of a 3 mg dose of the GnRH antagonist Cetrorelix in preventing premature LH surges: report of two large multicentre, multinational, phase IIIb clinical experiences. Reprod Biomed Online 2003; 6:432–438.
55. Lee TH, Wu MY, Chen HF, Chen MJ, Ho HN, Yang YS. Ovarian response and follicular development for single-dose and multiple-dose protocols for gonadotropin-releasing hormone antagonist administration. Fertil Steril 2005; 83:1700–1707.
56. Wilcox J, Potter D, Moore M, Ferrande L, Kelly E. Cap IV investigator group prospective, randomized trial comparing cetrorelix acetate and ganirelix acetate in a programmed, flexible protocol for premature luteinizing hormone surge prevention in assisted reproductive technologies. Fertil Steril 2005; 84(1): 108–117.
57. Ludwig M, Katalinic A, Banz C, et al. Tailoring the GnRH antagonist Cetrorelix acetate to individual patients' needs in ovarian stimulation for IVF: results of a prospective, randomized study. Hum Reprod 2002; 17:2842–2845.
58. Kolibianakis EM, Albano C, Kahn J, et al. Exposure to high levels of luteinizing hormone and estradiol in the early follicular phase of gonadotropin-releasing hormone antagonist cycles is associated with a reduced chance of pregnancy. Fertil Steril 2003; 79:873–880.

59. Al-Inany H, Aboulghar MA, Mansour RT, Serour GI. Optimizing GnRH antagonist administration: meta-analysis of fixed versus flexible protocol. Reprod Biomed Online 2005; 10:567–570.
60. Kolibianakis EM, Albano C, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Initiation of gonadotropin-releasing hormone antagonist on day 1 as compared to day 6 of stimulation: effect on hormonal levels and follicular development in in vitro fertilization cycles. J Clin Endocrinol Metab 2003; 88:5632–5637.
61. Olivennes F, Fanchin R, Bouchard P, Taieb J, Frydman R. Triggering of ovulation by a gonadotropin-releasing hormone (GnRH) agonist in patients pretreated with a GnRH antagonist. Fertil Steril 1996; 66:151–153.
62. Lubin V, Charbonnel B, Bouchard P. The use of gonadotrophin-releasing hormone antagonist in polycystic ovarian disease. Baillieres Clin Obstet Gynaecol 1998; 12:607–618.
63. Hwang JL, Seow KM, Lin YH, et al. Ovarian stimulation by concomitant administration of Cetrorelix acetate and HMG following Diane-35 pre-treatment for patients with polycystic ovary syndrome: a prospective randomized study. Hum Reprod 2004; 19:1993–2000.
64. Elizur SE, Aslan D, Shulman A, Weisz B, Bider D, Dor J. Modified natural cycle using GnRH antagonist can be an optional treatment in poor responders undergoing IVF. J Assist Reprod Genet 2005; 22:75–79.
65. Nikolettos N, Al-Hasani S, Felberbaum R, et al. Gonadotropin-releasing hormone antagonist protocol: a novel method of ovarian stimulation in poor responders. Eur J Obstet Gynecol Reprod Biol 2001; 97, 202–207.
66. Akman MA, Erden HF, Tosun SB, Bayazit N, Aksoy E, Bahceci M. Comparison of agonistic flare-up-protocol and antagonistic multiple dose protocol in ovarian stimulation of poor responders: results of a prospective randomized trial. Hum Reprod 2001; 16:868–870.
67. Schmidt DW, Bremner T, Orris JJ, Maier DB, Benadiva CA, Nulsen JC. A randomized prospective study of microdose leuprolide versus ganirelix in in vitro fertilization cycles for poor responders. Fertil Steril 2005; 83:1568–1571.
68. Mohamed KA, Davies WA, Allsopp J, Lashen H. Agonist "flare-up" versus antagonist in the management of poor responders undergoing in vitro fertilization treatment. Fertil Steril 2005; 83:331–335.
69. Cheung LP, Lam PM, Lok IH, et al. GnRH antagonist versus long GnRH agonist protocol in poor responders undergoing IVF: a randomized controlled trial. Hum Reprod 2005; 20:616–621.
70. Filicori M, Cognigni GE, Samara A, et al. The use of LH activity to drive folliculogenesis: exploring uncharted territories in ovulation induction. Hum Reprod Update 2002; 8:543–557.
71. Kolibianakis EM, Zikopoulos K, Schiettecatte J, et al. Profound LH suppression after GnRH antagonist administration is associated with a significantly higher ongoing pregnancy rate in IVF. Hum Reprod 2004; 19:2490–2496.
72. Cedrin-Durnerin I, Grange-Dujardin D, Laffy A, et al. Recombinant human LH supplementation during GnRH antagonist administration in IVF/ICSI cycles: a prospective randomized study. Hum Reprod. 2004; 19:1979–1984.

73. Griesinger G, Schultze-Mosgau A, Dafopoulos K, et al. Recombinant luteinizing hormone supplementation to recombinant follicle-stimulating hormone induced ovarian hyperstimulation in the GnRH-nt multiple-dose protocol. Hum Reprod 2005; 20:1200–1206.
74. Barmat LI, Chantilis SJ, Hurst BS, Dickey RP. A randomized prospective trial comparing gonadotropin-releasing hormone (GnRH) antagonist/recombinant follicle-stimulating hormone (rFSH) versus GnRH-agonist/rFSH in women pre-treated with oral contraceptives before in vitro fertilization. Fertil Steril 2005; 83:321–330.
75. Fanchin R, Salomon L, Castelo-Branco A, Olivennes F, Frydman N, Frydman R. Luteal estradiol pre-treatment coordinates follicular growth during controlled ovarian hyperstimulation with GnRH antagonists. Hum Reprod 2003, 18: 2698–2703.
76. de Ziegler D, Brioschi PA, Benchaa C, et al. Programming ovulation in the menstrual cycle by a simple innovative approach: back to the future of assisted reproduction. Fertil Steril 1999; 72:77–82.
77. Shapiro DB, Mitchell-Leef D, Carter M, Nagy ZP. Ganirelix acetate use in normal- and poor-prognosis patients and the impact of estradiol patterns. Fertil Steril 2005; 83:666–670.
78. Tavaniotou A, Albano C, Smitz J, Devroey P. Comparison of LH concentrations in the early and mid-luteal phase in IVF cycles after treatment with HMG alone or in association with the GnRH antagonist cetrorelix. Hum Reprod 2001, 16:663–667.
79. Albano C, Smitz J, Tournaye H, Riethmuller-Winzen H, Van Steirteghem A, Devroey P. Luteal phase and clinical outcome after human menopausal gonadotrophin/gonadotrophin releasing hormone antagonist treatment for ovarian stimulation in in vitro fertilization/intracytoplasmic sperm injection cycles. Hum Reprod 1999; 14:1426–1430.
80. Albano C, Grimbizis G, Smitz J, et al. The luteal phase of nonsupplemented cycles after ovarian superovulation with human menopausal gonadotropin and the gonadotropin-releasing hormone antagonist cetrorelix. Fertil Steril 1998; 70:357–359.
81. Beckers NG, Macklon NS, Eijkemans MJ, et al. Nonsupplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in in vitro fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. J Clin Endocrinol Metab 2003; 88: 4186–4192.
82. Lin Y, Kahn JA, Hillensjo T. Is there a difference in the function of granulose-luteal cells in patients undergoing in vitro fertilization either with gonadotrophin-releasing hormone agonist or gonadotrophin-releasing hormone antagonist? Hum Reprod 1999; 14:885–888.
83. Ragni G, Vegetti W, Baroni E, et al. Comparison of luteal phase profile in gonadotrophin stimulated cycles with or without gonadotrophin-releasing hormone antagonist. Hum Reprod 2001; 16:2258–2262.

84. Gonen Y, Balakier H, Powell W, Casper RF. Use of gonadotropin-releasing hormone agonist to trigger follicular maturation for in vitro fertilization. J Clin Endocrinol Metab 1990; 71:918–922.
85. Emperaire JC, Ruffie A. Triggering ovulation with endogenous luteinizing hormone may prevent the ovarian hyperstimulation syndrome. Hum Reprod 1991, 6:506–510.
86. Itskovitz J, Boldes R, Levron J, Erlik Y, Kahana L, Brandes JM. Induction of preovulatory luteinizing hormone surge and prevention of ovarian hyperstimulation syndrome by gonadotropin-releasing hormone agonist. Fertil Steril 1991; 56: 213–220.
87. Lewit N, Kol S, Manor D, Itskovitz-Eldor J. Comparison of GnRH analogs and HCG for induction of ovulation and prevention of ovarian hyperstimulation syndrome (OHSS): a case control study. Hum Reprod 1996, 11:1399–1402.
88. Fauser BC, de Jong D, Olivennes F, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. J Clin Endocrinol Metab 2002; 87:709–715.
89. Humaidan P, Bredkjaer HE, Bungum L, et al. GnRH agonist (Buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: a prospective randomized study. Hum Reprod 2005; 20:1213–1220.
90. Itskovitz J, Kol S, Mannaerts B. Use a single bolus of GnRH agonist Triptorelin to trigger ovulation after GnRH antagonist ganirelix treatment in women undergoing ovarian stimulation for assisted reproduction, with special reference to the prevention of ovarian hyperstimulation syndrome: preliminary report. Hum Reprod 2000; 15:1965–1968.
91. Kolibianakis EM, Schultze-Mosgau A, Schroer A, et al. A lower ongoing pregnancy rate can be expected when GnRH agonist is used for triggering final oocyte maturation instead of HCG in patients undergoing IVF with GnRH antagonists. Hum Reprod. In press.
92. Antoine JM. GnRH antagonists in insemination: can we avoid weekends? J Gynecol Obstet Biol Reprod (Paris) 2004; 33:3S50–3S52.
93. Gomez-Palomares JL, Julia B, Acevedo-Martin B, Martinez-Burgos M, Hernandez ER, Ricciarelli E. Timing ovulation for intrauterine insemination with a GnRH antagonist. Hum Reprod 2005; 20:368–372.
94. Williams RS, Hillard JB, De Vane G, et al. A randomized, multicenter study comparing the efficacy of recombinant FSH vs recombinant FSH with Ganirelix during superovulation/IUI therapy. Am J Obstet Gynecol 2004; 191:648–651.
95. Olivennes F, Mannaerts B, Struijs M, Bonduelle M, Devroey P. Perinatal outcome of pregnancy after GnRH antagonist (ganirelix) treatment during ovarian stimulation for conventional IVF or ICSI: a preliminary report. Hum Reprod 2001; 16:1588–1591.
96. Ludwig M, Riethmuller-Winzen H, Felberbaum RE, et al. Health of 227 children born after controlled ovarian stimulation for in vitro fertilization using the luteinizing hormone-releasing hormone antagonist Cetrorelix. Fertil Steril 2001; 75:18–22.
97. Olivennes F, Cunha-Filho JS, Fanchin R, Bouchard P, Frydman R. The use of GnRH antagonists in ovarian stimulation. Hum Reprod Update 2002; 8:279–290.

6

Ultrasonography in In Vitro Fertilization

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Imaging has become such an integral part of clinical care in the assisted reproductive technologies that it is difficult to imagine how in vitro fertilization (IVF) was done before we had the ability to visualize the ovaries and uterus easily. Recall that IVF was once done using laparoscopic retrieval of oocytes following ovarian stimulation cycles monitored only by hormonal assay of systemic estradiol levels, that embryos were transferred back into a uterus when we had no real idea about the physiologic status of the endometrium, and only a clinical touch was used to guide the placement of the embryo transfer catheter. Easily accessible, and easy-to-use, ultrasonographic imaging in the hands of the individuals performing the assisted reproductive technology (ART) procedures has delivered us from those uncertainties. The quality and quantity of the information we received from the ultrasonographic images that are now an essential part of every procedure have been a very important aspect of the incredible increases in ART success rates we have seen over the past decade. It is important to remember that the integration of enhanced understanding of anatomy, physiology, endocrinology, and pathology we have gained with imaging in the patients undergoing IVF are as important as the fantastic increase in knowledge in the embryo laboratories. The confluence of technologies

we now used in ART care have greatly increased the probabilities of successful pregnancies for our patients.

The purpose of this chapter is to describe the primary uses of ultrasound imaging in IVF and to identify some promising new areas where imaging has the potential to enhance our understanding in assisted reproduction. The essentials of ultrasonography in IVF are in monitoring the course of ovarian stimulation protocols, visually guided retrieval of oocytes, assessment of the endometrium, and visually guided embryo transfer. Each of these areas also provides a springboard for new research areas which may be incorporated into clinical care. Awareness of new frontiers is essential to progress in ART and in understanding the changes that will surely come. We rely so heavily on imaging in general gynecology, infertility workup, and early obstetrical care that it becomes challenging to narrow our focus to only IVF; however, with the general caveat that ultrasonography has forever changed our understanding of female reproduction, my goal is to provide a synopsis of imaging in IVF integrated into a framework within which we provide the highest quality of care for the patients who require ART to complete their families.

OVARIAN ASSESSMENT

Monitoring the Course of Ovarian Stimulation

Ovarian stimulation protocols vary tremendously and have evolved from fairly simplistic administration of exogenous hormones derived from urinary sources to quite sophisticated blends of gonadotrophin-releasing hormone (GnRH) analogs, recombinant follicle-stimulating hormone (FSH), luteinizing hormone (LH), and other compounds. The common denominator in all ovarian stimulation protocols is that ultrasonography is used to determine their effects on the ovaries of each patient. All the protocols have been designed to override the physiologic mechanism of selection of a single dominant follicle, obviate atresia in the cohort of follicles recruited into the follicular wave, and foster and sustain the development of many follicles to an imminently pre-ovulatory state so that properly matured oocytes may be retrieved for IVF. Ultrasonography is essential in determining the numbers and fates of individual follicles stimulated by exogenous gonadotrophins. Toward this end, the follicular response of each woman to the stimulation protocol and the number of oocytes desired and clinical assessment of the risk of ovarian hyperstimulation will dictate increasing or decreasing daily doses of gonadotrophins. It is important to note that the expected linear relationship between circulating estradiol concentrations and follicular diameter may not exist during ovulation induction. Similarly, we understand that all follicles probably do not contribute equally to the concentrations in the systemic circulation.

In all the ART ovarian stimulation protocols, regardless of their technical complexity, the timing for human chorionic gonadotrophin (hCG) or recombinant LH administration is critical to establish a time for oocyte retrieval which will yield the highest quality of oocytes in the proper stage of development with the highest probability of fertilization. Although transabdominal ultrasonography has been used, transvaginal ultrasonography (TVUS) is the best means that we have to follow the course of follicular growth and development (1,2). With TVUS, we have a rapid, non-invasive, and highly visual approach to following the fates of individual follicles and cohorts of follicles. When we combine our knowledge of natural ovarian physiology with concomitant assessment of circulating estradiol concentrations and oocyte development, we may predict the optimal timing for induction of the final stages of folliculogenesis and oogenesis and oocyte retrieval (3–5). The relationships between follicle size and oocyte maturity remain not particularly well elucidated; however, the oocyte maturity certainly plays a role in the ability of the resulting embryos to develop to the blastocyst stage (6–8).

hCG is usually administered to trigger the final phases of follicular maturation when the largest follicle first attains a predetermined diameter (e.g., 18–20 mm). The time of hCG administrations varies in many programs based upon the individual clinician's feel for the stimulation cycle and laboratory logistics. Most commonly, 5000 or 10,000 IU hCG is administered. Oocyte retrieval for IVF is then typically scheduled for 30–34 hr thereafter. Many programs use only ultrasonographic monitoring to determine the course of ovarian stimulation and it has been demonstrated that including estradiol monitoring during the stimulation protocol seldom changed the timing of hCG administration and did not affect pregnancy rates or the risks of ovarian hyperstimulation syndrome (OHSS) (9).

The characteristics and appropriate sizes of follicles which produce mature oocytes ready for fertilization remain the subject of much controversy and research. Although we know that mature oocytes yield the highest fertilization rates (Fig. 1), through recent developments in the embryo laboratory, we know that in vitro maturation and fertilization are quite viable ideas in ART practice (10,11). The role of ultrasonography in vitro maturation (VM)–IVF protocols will very definitely revolve around the optimal timing of oocyte retrieval for optimal fertilization and cleavage rates (11–14). Ovulation has been reported from follicles as small as 14 mm and oocytes collected from small follicles may indeed fertilize. In a recent study, oocytes from follicles less than 10 mm in diameter and in vitro maturation were used to increase the number of transferable embryos (15). Through research in animal models, we know that there appears to be a correlation between computer-assisted ultrasound image attributes of follicles and the ability of the oocyte to fertilize; however, similar studies in humans have apparently not yet been completed (16).

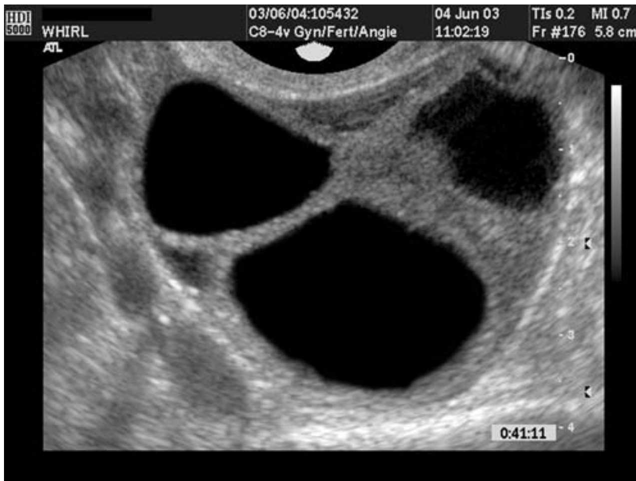

Figure 1 Image of an ovary with three dominant follicles visible in the plane of section. The image was acquired 24 hours prior to oocyte retrieval. The thick walls of the follicles are consistent with collection of oocytes with a high probability of fertilization.

Examination of growth rates for individual follicles may be a useful characteristic with which to predict the number of follicles which may develop during ovarian stimulation protocols. This information is equally important when assessing the risks of ovarian hyperstimulation. In the past, follicular growth rates during induced cycles were observed to be faster than those of natural cycles (17). However, a mathematical equation developed to equate follicular growth rate to follicular age was used to conclude that the growth rates of individual follicles in spontaneous cycles were similar to those recruited by human menopausal gonadotrophin therapy (18). Reduced growth rates of follicles in cycles where a pregnancy was established led to the conclusion that growth rate was a more useful characteristic for prediction of ovulation than follicular diameter (19). Follow-up work does not appear to have been done. It will be logistically challenging to combine daily detailed ultrasound measurements of individually mapped follicles with per follicle outcomes from the embryo laboratory and final pregnancy outcomes. However, the rationale that follicular growth rates may be more accurate in predicting the actual maturity of the ova is intriguing. Recent detailed studies on follicular growth have shown that follicles grow at approximately 1.5 mm per day regardless of whether they developed during natural menstrual cycles, oral contraceptive cycles, or during ovarian stimulation (20,21). These data fit well with a new mathematical model developed to predict the ovarian response to superstimulation

protocols and based upon daily data on follicular growth rates (22,23). It is evident that new imaging-based studies are required.

Assessment of Ovarian Follicular Reserve

Changes in demographic trends in the age at first pregnancy in our times have combined to yield more and more women seeking pregnancy when they are older and less fertile. Numerous studies in recent years have demonstrated that fertility declines progressively as age advances. In IVF, the main focus of attention is on assessment of what is termed the ovarian reserve. Ultrasonography is now being used to investigate follicular dynamics in aging women as are detailed endocrine-based tests (24). A decrease in the ovarian reserve, or number of follicles capable of being stimulated, is a primary reason for declining fertility. Similarly, the ovarian response to exogenous gonadotrophin stimulation also decreases, but the range of individual variation is extremely wide and it is well known that age is only a rough guesstimate of the ovarian reserve and hence the ovarian stimulation response.

There are several tests of "ovarian reserve" that include clomiphene citrate challenge and the GnRH agonist stimulation tests (24–27). Ovarian biopsy is available, although it remains controversial (24,28). The endocrine tests offer prognostic information valuable in the counseling of aging infertile women. However, there is much recent evidence to suggest that ultrasonography may be used to estimate the number of antral follicles at specific times of the menstrual cycle and provide additional useful information of clinical relevance (29–32). Ultrasound assessments take place using antral follicle counts or measurement of ovarian volume. Early follicular-phase antral follicle counts, typically done on days 3 to 7 post-menstruation, may be used to predict the number of follicles likely to develop during ovarian stimulation with exogenous gonadotrophins (31,33–36). Women having fewer than five follicles under 10 mm in diameter before ovarian stimulation begins have a relatively poor prognosis for success (35). Studies to determine the extent to which antral follicle counts correlate with endocrinologic measures of ovarian reserve (e.g., cycle day 3 FSH and estradiol concentrations) remain to be widely confirmed (30). Ovarian volume assessments are based on the presumption that there is a significant correlation between the population of primordial follicles remaining in the ovary and the volume of the ovary, measured using either two- or three-dimensional ultrasonography (27,29,33,37). A very clear relationship between decreased ovarian volume and antral follicle counts and advancing age combined with increased FSH has been demonstrated (26,30). Although there remains a good deal of work yet to do in order to standardize the imaging based assessments, ultrasonography remains an important aspect of ovarian reserve estimation and prediction of the probability of a successful ovarian stimulation cycle (35).

Ovarian Hyperstimulation Syndrome

OHSS is a potentially serious complication of ovarian stimulation with exogenous gonadotrophins. The risk of serious disease is much higher when exogenous gonadotrophins are employed. In women with the disorder, transvaginal or transabdominal ultrasonography often demonstrates grossly enlarged ovaries containing numerous large follicular cysts with thin, highly echogenic borders, and dramatically increased local blood flow (38). The ovaries may enlarge to diameters in excess of 10 cm, and echotexture interpreted as intrafollicular hemorrhage in some of the large cysts frequently may be observed. Serial TVUS during ovarian stimulation cycles and careful tailoring of the dose of exogenous gonadotrophins has helped to limit the risk of OHSS (38,39). Clinicians take an active role in the prevention of OHSS by aborting the treatment cycle and cryopreserving the embryos for later, or replacement of a single embryo when excessive numbers of preovulatory follicles develop in association with markedly elevated serum estradiol concentrations and the risk of OHSS is high (40). When OHSS does occur, torsion of an enlarged ovary is a complication that must be kept in mind. When torsion is suspected, color flow Doppler imaging can help to establish an early and accurate diagnosis (41,42).

Computer-Assisted Ultrasonographic Imaging of Follicular Development

New work in application of computer-assisted image analysis is demonstrating that ultrasound images have the potential to aid in the identification of healthy versus atretic follicles in natural and ovarian stimulation cycles (22,43,44) (Fig. 2). Physiologically dominant ovarian follicles are identifiable by ultrasonography at approximately day 7 post-menstruation in unstimulated cycles (22), and ovulatory and non-ovulatory follicles are identifiable in ovulation induction cycles (43). The image attributes of ultrasonographic images of normal preovulatory follicles include thick, low-amplitude walls and a gradual transformation zone at the fluid–follicle interface. The walls of preovulatory follicles are characterized by increased heterogeneity, increased wall breadth, and a more gradual transformation at the fluid–follicle wall interface. Atresia is characterized by thin walls, high numerical pixel value (bright) signals, and highly variable signals from the follicular fluid. Evaluation of the acoustic characteristics indicative of viability and atresia is an active area of research that has profound implications for development of safer and more effective ovarian stimulation protocols.

ULTRASOUND-GUIDED OOCYTE RETRIEVAL

The most visible use of ultrasound imaging in IVF has been the tremendous advance facilitated by transvaginal retrieval of oocytes (45–55). Oocyte

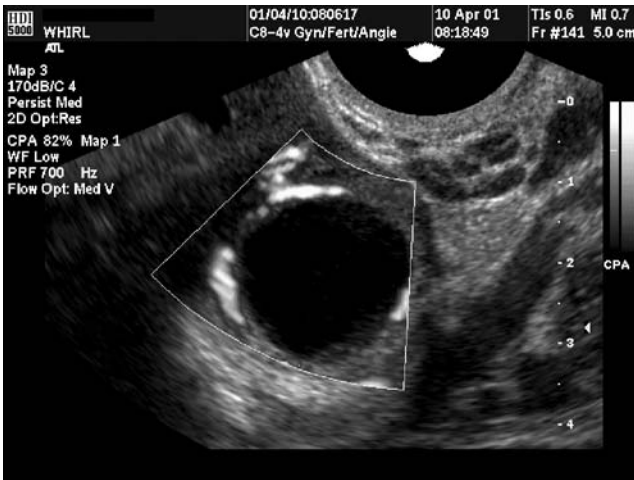

Figure 2 Power-flow Doppler image of a dominant preovulatory follicle showing the perifollicular vascularization consistent with follicular maturity.

retrieval was a procedure-limiting step when IVF was first done. Retrievals were done laparoscopically or using ultrasound guidance from transurethral, transvesicular, or transabdominal approaches (48,51,55–58). The advent of transvaginal transducers and concerted efforts to develop effective, accurate tracking of the needles used for follicle aspiration was probably the single most important step in making IVF as safe and effective as it is today (Fig. 3) (45,53–55,59–63).

Retrieval of oocytes in IVF cycles is now routinely performed under TVUS guidance (49). An aspirating needle is introduced through a guide attached to a transvaginal probe and is inserted into first one ovary, then the other, via the vaginal fornices. Almost all aspiration needles now in common use have a small band of highly reflective surface near the tip of the needle to facilitate ease of visualization as the needle enters the ovary and once it is in the follicles. The path of the needle as it is guided into each ovarian follicle may be accurately defined by a biopsy guideline imposed on the ultrasound screen, although, the highly reflective walls of the needle make identifying its path quite easy in most cases. The needle tip can be observed directly as it is maneuvered within the ovaries and into each follicle. The follicular fluid containing the oocyte/cumulus complex is then aspirated by application of gentle suction. The walls of the follicle collapse as the fluid is aspirated and the needle moved within the follicle to ensure that all the follicular fluid is withdrawn.

There are two main types of aspiration needles used for oocyte retrieval, single and double lumen needles. Single lumen needles typically

have a smaller diameter and tend to cause less discomfort (49). In many, if not most, IVF centers follicle aspirations are done using single lumen needles and no follicle flushing. The double lumen needles were developed for a technique involving constant infusion of oocyte collection media into the follicle at the same time as the follicular fluid is being removed. The double lumen flushing technique is thought to increase the turbulence within the follicle, assist in dislodging the oocyte–cumulus complex from the follicle wall, and increase the chances of oocyte collection. A single lumen needle flushing technique may also be used. In this technique, all the follicular fluid is first aspirated from the follicle and the follicle is then refilled with collection medium and re-aspirated. A back-and-forth motion on the plunger of the infusion syringe may be used to increase the turbulence of flow which may be easily visualized on the ultrasound screen. No significant differences were found in the number of oocytes collected in either a prospective, randomized trial or a retrospective examination of 2378 cases and the time required for retrieval in women whose follicles were flushed was increased (49,64,65).

Unsuccessful oocyte retrieval following apparently normal ovarian stimulation reportedly occurs in 1–7% of cycles—the so-called "empty follicle syndrome." The etiology appears to be multifactorial and may involve both technical and biological mechanisms (49,66).

The complication rates of oocyte retrieval are reportedly extremely low and almost all procedures are performed under conscious sedation on an outpatient basis (52,60,61,67–72).

ASSESSMENT OF THE ENDOMETRIUM AT EMBRYO TRANSFER

Endometrial Thickness and Pattern for Assessing Endometrial Receptivity

Ultrasonography has been used, with varying degrees of success, to correlate the probability of pregnancy in ovarian stimulation–ovulation induction cycles and IVF cycles (73–77). Most imaging studies have been attempting to predict the probability of implantation. A thicker endometrium was observed on the day of oocyte retrieval in women who conceived during that cycle (74). The IVF pregnancy rate increased in cycles when the endometrium was >9 mm but <14 mm (75). In another study, no correlation was observed among endometrial pattern or thickness and estradiol levels, number of oocytes retrieved, or progesterone level on the day of embryo transfer; however, the authors appeared to appreciate the pattern of the endometrium on the day of hCG administration, but stated that pattern assessment was of no value (78). In another IVF study, the endometrium on the day before embryo transfer was nearly 2 mm thicker in women who conceived (10.2 mm) than in those who did not (8.6 mm) (79). Only two pregnancies were reported when the endometrial thickness was less than

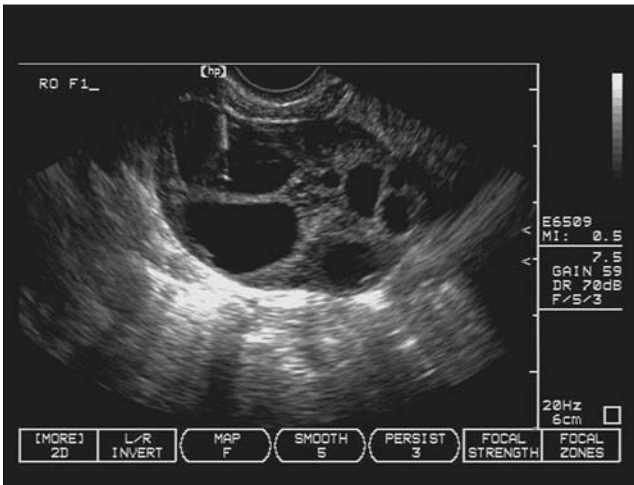

Figure 3 Ultrasound image taken during oocyte retrieval. The highly echogenic band around the distal end of the needle and the tip of the follicle aspiration needle are visualized in the superior-most follicle. The needle is maneuvered within the ovary to aspirate all follicles. *Source*: Image courtesy of Dr. Roger Stronell.

7.5 mm, and no pregnancies were observed when the endometrial thickness on the day of embryo transfer was less than 5 mm. However, no differences were observed in endometrial thickness among women who conceived compared to those who did not in a similar study (80). Subsequently, a more favorable outcome has been suggested when embryos were transferred when the endometrial thickness was greater than 9 mm and a "triple-line" pattern was observed (81). This observation was supported by a retrospective analysis in which the pregnancy rate was significantly higher in women who exhibited a triple-line pattern than in those with other endometrial patterns (Fig. 4) (82). These contradictory reports and the apparent lack of correlation between ultrasonographic endpoints and histologic staging of the endometrium in women undergoing IVF can be interpreted to suggest that ultrasonography using simple measurements is simply not yet sensitive enough to be useful in predicting endometrial receptivity and the probability of implantation with the exception of a strong negative correlation when the endometrium is thin (81,83,84). It is also possible that inconsistencies in the day on which measurements were done among the many studies and measurement techniques have played a role in our seeming inability to interpret the data. Consensus among studies is that implantation may occur as long as the endometrial thickness is greater than 6 mm, although there is a single case report of a pregnancy established when the endometrium measured 4 mm (74,85–87).

Collections of fluid are sometimes found within the uterine lumen on the day of embryo transfer (88) (Fig. 5). In a retrospective analysis of case records, approximately 5% of cycles were compromised by the presence of lumen fluid accumulation at some time during the IVF cycle procedures, and in 2% of the cases the fluid accumulations persisted until the day of embryo transfer. The pregnancy rate among women with fluid accumulations was markedly lower than those who did not exhibit intralumenal fluid. Interestingly, fluid accumulations were found in almost three times as many women with tubal factor infertility compared with other causes (88). Although lumenal fluid collection does not appear to be a common problem in IVF cycles, it does appear to have a negative impact on implantation and pregnancy rates.

Spectral Doppler and Color Flow Doppler Ultrasonography

The history of Doppler ultrasonography of the uterine arteries in the literature is confusing because many reports failed to differentiate between spectral Doppler and color flow Doppler imaging. Early studies tend to be based on spectral Doppler examinations, which are a means of evaluating the resistance to blood flow using calculations of the pulsatility index (PI), resistance index (RI), $V_{\max}$, or the systolic-to-diastolic ratio (S/D ratio). Color flow Doppler and power flow Doppler imaging are means of turning motion, either toward or away from the transducer in the case of color flow

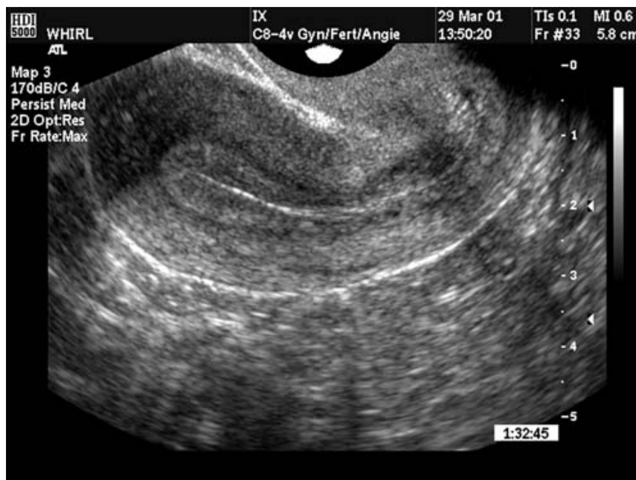

Figure 4 Midsagittal view of the uterus. The cervix is to the right of the image and the fundus is to the left. The endometrium is well demarcated and shows a pronounced, thick "triple-line" pattern associated with a higher probability of implantation following embryo transfer.

Doppler, or motion in any direction in the case of power flow Doppler, into a visually detectable color overlay on the two-dimensional ultrasound image (Fig. 2) (89).

Initially, attempts to determine if evaluation of blood flow in the uterine arteries could be useful were based on RI calculations to look for differences in uterine receptivity, where in a small series of women, no differences were found between women who conceived and those who did not (90). When the PI of uterine arteries was examined and data were grouped into low, medium, and high categories, no differences were found between cycles where women either conceived or did not; however, no pregnancies were established in the women with high PI values (91). Elevated PI, as a measure of impedance to vascular flow in the uterine artery, was associated with a significantly lower pregnancy rate (92). However, no differences in uterine artery PI were observed between conception and non-conception cycles (93). A study assessing RI of the uterine arteries was inconclusive, except that absent or low diastolic flow was associated with failure to conceive (93). Uterine artery vascular impedance measured by RI was not found to be useful for predicting the probability of pregnancy, but if the PI values were found to be greater than 0.79 before hCG administration, poor uterine vascular perfusion was assumed (94). A subsequent study reported the PI and RI in the uterine arteries to be lower in conception cycles, and the authors suggested that a PI greater than 3.3 and an RI greater than 0.95 before embryo transfer were associated with a low probability of conception (95). Furthermore, a study of women undergoing cycles in which embryos were produced using ICSI reported no demonstrable differences in PI on the day of embryo transfer between conception and non-conception cycles (96).

In a critical review of the literature prior to 1996, some ultrasonographically detectable criteria were observed to be associated with negative pregnancy outcomes; however, no prognostic value was observed in any measurement of vascular perfusion (97). A more recent study appears to confirm the results that women who conceived exhibited lower PI than those who did not (98).

Studies that evaluated endometrial perfusion on the day of hCG administration consistently reported that values for PI and V_{max} were not different, irrespective of whether or not conception was established (95,97,99–101). However, when only the color flow data were examined, absence of detectable subendometrial vascular flow, indicative of poor vascular penetration, was associated with failure of implantation (99). Power flow Doppler ultrasonography was subsequently used to examine women whose endometrial thickness was >10 mm. Intra-endometrial flow calculations of the maximal area that showed evidence of motion indicative of vascular flow of $<5\,mm^2$ were associated with a lower pregnancy rate (100). Subsequently, a high degree of endometrial perfusion visualized using three-dimensional ultrasonography was thought to indicate a more favorable endometrium (102). The most recent study in this line of inquiry

concluded that spiral arterial flow and uterine artery flows were not different between pregnant and non-pregnant women; however, if spiral arterial flow could not be detected, there was no probability of conception (101).

Imaging-Based Uterine Scoring System

An imaging-based scoring system to predict uterine sensitivity has been proposed which appears to be based on an earlier uterine biophysical profile system (103,104). The scoring system was designed by assigning "points" for various criteria and then adding the cumulative columns. Comparisons of uterine scores in conception with non-conception cycles demonstrated no differences in any criteria measured, including endometrial thickness, endometrial pattern, PI, RI, color Doppler, and other vascular indices. Development of the scoring system appears to have ceased.

Three-Dimensional Imaging of the Endometrium

Three-dimensional (3D) ultrasonography first became available in the late 1990s and 3D is now a part of almost all high-end imaging systems. There are several methods used to provide 3D information and there are no studies comparing the same endpoints with different imaging systems (105). The prospects for predicting the probability of implantation in IVF programs have now extended into 3D exploration of endometrial receptivity (106).

When endometrial volumes were compared among women who conceived and those who did not, pregnancy and implantation rates were significantly lower in women with volumes of less than 2 mL, and no pregnancies were established when endometrial volumes were less than 1 mL. A contemporary study found no relationship between 3D volume of the endometrium and conception (106,107). Nor was a correlation found among estradiol levels, endometrial thickness, or endometrial volume, leading the authors to conclude that there was no predictive value for conception in assessing endometrial volume. Endometrial thickness and endometrial volumes were not correlated with probability of pregnancy; however, 3D power flow Doppler indices used to measure endometrial perfusion may have some predictive value (101,102,108,109). Spiral artery blood flow measurements in 3D had positive predictive value when performed on the first day of ovarian stimulation, whereas women who became pregnant had lower RI and a higher 3D flow index than those that did not (101,102). Taken together, these observations provide rationale for further investigation, although it is clear that a predictive index is beyond the limits of our current technology.

Motion Analysis

Motion analysis, or direct measurement of subendometrial contractions, is a method of evaluating the endometrium based on the observation that the

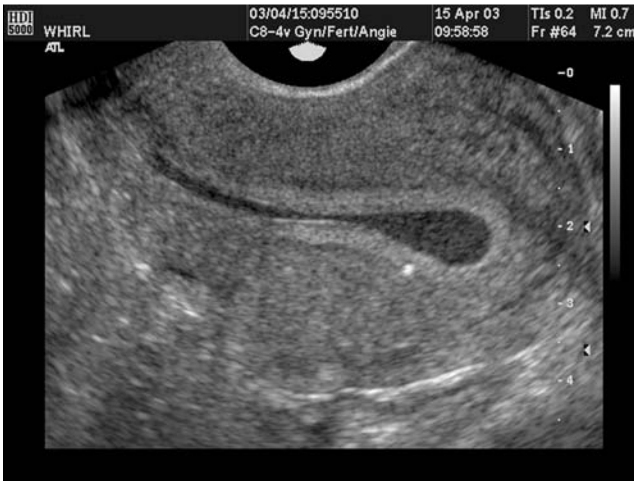

Figure 5 Midsagittal view of a uterus with a pronounced intraluminal fluid collection. The cervix is visualized to the left of the image and the fundus to the right. Fluid collections on the day of embryo transfer are associated with a very low probability of pregnancy.

uterus and endometrium are in constant motion (110–117). Objective assessment of these contractions has now been applied to assisted reproduction cycles (118–120). A computer is interfaced with an ultrasound instrument, digital frames are acquired, and the pixels comprising the endometrial image along a single line are isolated (Fig. 6). A line is serially acquired from images taken one or two times per second over a 5–10-min period, the pixel data from the line are concatenated, and the result is displayed as a graph of the velocity and amplitude of endometrial contractions.

Endometrial contractions may have a predictive effect on the probability of pregnancy in IVF cycles (114,121). Women with a higher frequency of uterine contractions were found to have lower pregnancy rates (119). However, contradictory evidence has been reported (122). Exogenous progesterone has demonstrably reduced uterine contractility on the day of embryo transfer and it has been hypothesized that progesterone supplementation before embryo transfer may improve endometrial receptivity by lowering the possibility that embryos might be expelled from the uterus by contractions (123–125). Uterine contractility at the time of blastocyst transfer was lower and reached a nadir 7 days after hCG administration in IVF cycles. The low amplitude and frequency of contractions are hypothesized to facilitate blastocyst implantation (126). The effects of progesterone on uterine contractions have been demonstrated by the observation that higher progesterone concentrations correlated with lower amplitude and frequency uterine contractions (115,125). It was suggested

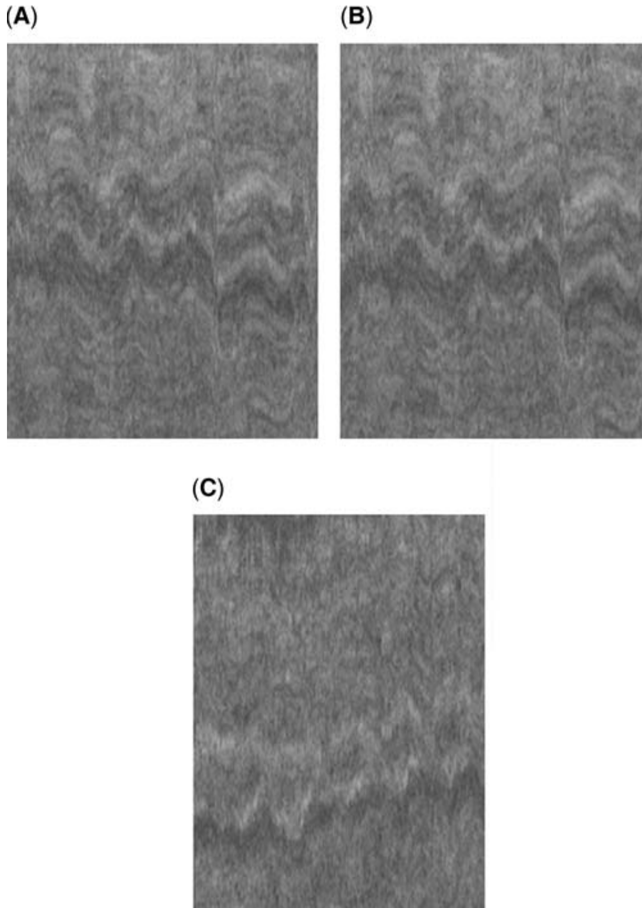

Figure 6 Ultrasound-based motion analysis images (A–C) of 1.5 minutes in duration, showing individual women with slow (A: 3 contractions per minute), medium (B: 5 contractions per minute), and fast (C: 7.5 contractions per minute). The amplitude and frequency of the contractions are easily visualized. *Source*: From Ref. 159.

that progesterone could be administered to reduce endometrial contractions and have a positive impact on pregnancy rates, although this hypothesis does not appear to have been critically tested (123).

ULTRASOUND-GUIDED EMBRYO TRANSFER

Ultrasonographic imaging is now being used to guide the placement of the embryo transfer catheter in an effort to facilitate optimal embryo placement

and enhance the probability of a successful pregnancy (127–137). Transabdominal ultrasound guidance is a more common means of directing the embryo transfer catheter, however, transvaginal scanning may also be used (134,138). It is also important to note that ultrasound guidance of embryo replacement does not prevent the establishment of an ectopic gestation (139). The recent advent of easy-to-use and relatively inexpensive 3D ultrasonography has facilitated a new wave of enquiry into the utility of 3D imaging to guide the embryo transfer catheter (136,140). Early impressions are that 3D imaging may be beneficial in identifying the site of optimal embryo placement with respect to anatomic variations in individual women.

Clinical and laboratory preparations for embryo transfer are the same, regardless of whether the transfer is to be ultrasound guided or not. Patients are placed in the lithotomy position and the cervix exposed using a bivalve speculum. Mucus and secretions are removed using culture media and the tip of the transfer catheter is introduced into the os cervix. The addition of transabdominal ultrasound imaging simply involves placement of the transducer, typically using 3–4 MHz large aperture probe, on the lower abdomen and pelvis in the sagittal plane and imaging the full sagittal plane of the uterus and cervix through a full bladder window (Fig. 7) (133,141,142). Most standard embryo transfer catheters are easily visualized as a pair of highly echogenic lines within the cervix; however, a transfer catheter system has been developed to increase the ease of imaging (143). Once the catheter has been identified, the tip may be carefully guided through the uterine lumen using real-time imaging. Once the clinician attains the optimal place with in the uterus, the embryos are gently expelled (144). Opinion on exactly what optimal placement means is varied (145–148). The fluid droplet containing the embryos is visualized as a very small hypoechoic blip deposited at the tip of the transfer catheter. Transvaginal ultrasound guidance is done in a similar fashion, except that a probe designed for intracavitary use is introduced through the speculum and placed into contact with the anterior vaginal fornix (134,138). The transfer catheter is visualized and the tip guided to the optimal uterine location for embryo deposition.

There is a measure of controversy regarding the usefulness of ultrasonographic guidance during embryo transfer versus non-visually guided clinical touch (131,137,141,149–155). Some clinicians prefer to rely on ultrasound guidance for mock transfers in cycles before IVF and embryo transfer and clinical touch in the actual procedure, others use ultrasound guidance for all procedures, and still others make a decision regarding its use based on whether or not the transfer is likely to be classified as easy or difficult (137,144,155). Two recent meta-analyses and a subsequent randomized controlled trial have been interpreted to mean that transabdominal ultrasound guidance versus clinical touch for embryo transfer significantly increased the pregnancy rate, although the rates of miscarriage, ectopic pregnancy, and multiple pregnancy were not affected (154,156–158).

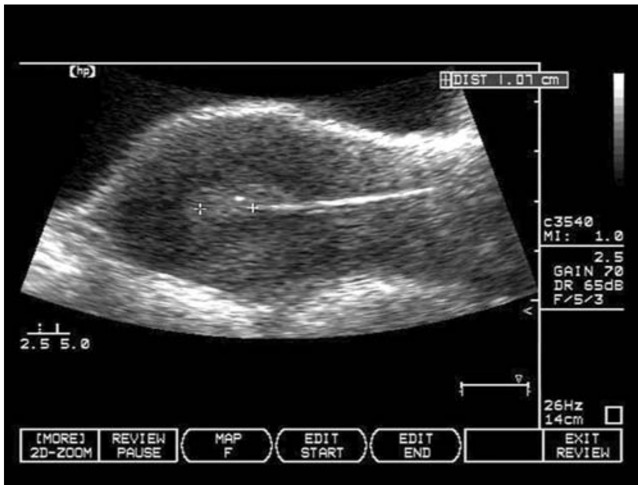

Figure 7 Midsagittal view of the uterus imaged with transabdominal ultrasonography during embryo transfer. The transfer catheter is visualized as the highly echogenic line in the middle of the uterus. Calipers (+) mark the distance from the tip of the catheter to the fundus. The image documents optimal replacement of the embryos. Source: Image courtesy of Dr. Roger Stronell.

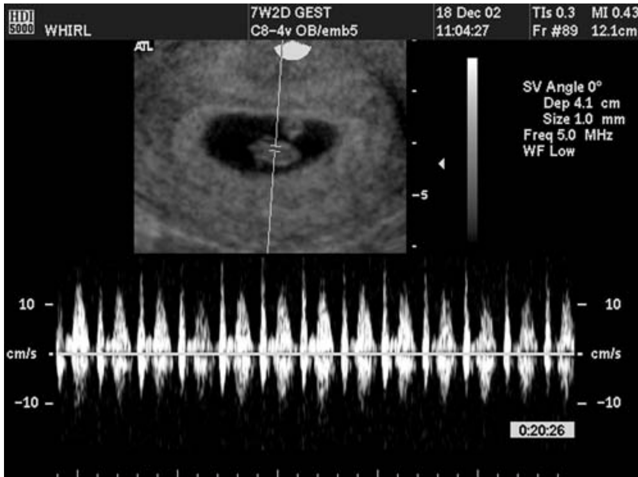

Figure 8 Gray-Scale and Spectral Doppler image of an embryo 5 weeks post transfer. The gestational sac, embryo and yolk sac are visualized in the top half of the image. the Dopplar gate is placed directly over the embroyomic heart and the resulting spectral doppler trace is seen in the lower half of the image reflecting cardiac activity.

CONCLUDING REMARKS

It seems strange to envision ART care without ultrasound imaging. The ability to see the effects of ovarian stimulation and optimize the protocol for individual patients, easy accessibility of the ovaries for oocyte retrieval, direct visualization of the endometrium at the time of embryo transfer, and embryo replacement under direct visual guidance are all easy to take for granted. Ultrasonography also provides direct visual access to all the events in human reproduction and allows us to elucidate biological phenomena approaching the natural state. One of the most joyous occasions we have in our work is when we are able to confirm pregnancy in our patients (Fig. 8). There is little that approaches the drama in the imaging suite when we can point out an embryo to our patients and listen to the heartbeat of their new life. When we combine the essential contributions to ART made by the dramatic advances made in the embryo laboratory with those made possible by advanced imaging technology, we enrich not only our scientific knowledge base, but also the lives of many couples who have struggled with infertility.

REFERENCES

1. Tarlatzis BC, Laufer N, Decherney AH. The use of ovarian ultrasonography in monitoring ovulation induction. J In Vitro Fert Embryo Transf 1984; 1:226–232.
2. Bonilla-Musoles F, Pardo G, Perez-Gil M, Serra V, Pellicer A. Abdominal ultrasonography versus transvaginal scanning: accuracy in follicular development evaluation and prediction for oocyte retrieval in stimulated cycles. J Clin Ultrasound 1989; 17:469–473.
3. Wittmaack FM, Kreger DO, Blasco L, Tureck RW, Mastroianni L Jr, Lessey BA. Effect of follicular size on oocyte retrieval, fertilization, cleavage, and embryo quality in in vitro fertilization cycles: a 6-year data collection. Fertil Steril 1994; 62:1205–1210.
4. Nagai S, Yasumizu T, Kasai T, Hirata S, Mizuno K, Kato J. Effect of oocyte retrieval from a small leading follicle in fixed-schedule in vitro fertilization program. J Obstet Gynaecol Res 1997; 23:165–169.
5. Requena A, Neuspiller F, Cobo AC. Endocrinological and ultrasonographic variations after immature oocyte retrieval in a natural cycle. Hum Reprod 2001; 16:1833–1837.
6. Huang FJ, Huang HW, Lan KC. The maturity of human cumulus-free oocytes is positively related to blastocyst development and viability. J Assist Reprod Genet 2002; 19:555–560.
7. Kitai H, Inagaki N, Kimura H. A study on the correlation between maturation and quality of human oocytes. Hum Cell 1997; 10:263–270.
8. Salha O, Nugent D, Dada T. The relationship between follicular fluid aspirate volume and oocyte maturity in in vitro fertilization cycles. Hum Reprod 1998; 13:1901–1906.

9. Lass A. Monitoring of in vitro fertilization–embryo transfer cycles by ultrasound versus by ultrasound and hormonal levels: a prospective, multicenter, randomized study. Fertil Steril 2003; 80:80–85.
10. Lin YC, Chang SY, Lan KC. Human oocyte maturity in vivo determines the outcome of blastocyst development in vitro. J Assist Reprod Genet 2003; 20:506–512.
11. Picton HM, Danfour MA, Harris SE. Growth and maturation of oocytes in vitro. Reprod Suppl 2003; 61:445–462.
12. Russell JB. Immature oocyte retrieval combined with in vitro oocyte maturation. Hum Reprod 1998; 13(suppl 3):63–70; discussion 71–75.
13. Russell JB. Immature oocyte retrieval with in vitro oocyte maturation. Curr Opin Obstet Gynecol 1999; 11:289–296.
14. Thornton MH, Francis MM, Paulson RJ. Immature oocyte retrieval: lessons from unstimulated IVF cycles. Fertil Steril 1998; 70:647–650.
15. Triwitayakorn A, Suwajanakorn S, Pruksananonda K. Correlation between human follicular diameter and oocyte outcomes in an ICSI program. J Assist Reprod Genet 2003; 20:143–147.
16. Vassena R, Adams GP, Mapletoft RJ. Ultrasound image characteristics of ovarian follicles in relation to oocyte competence and follicular status in cattle. Anim Reprod Sci 2003; 76:25–41.
17. DeCherney AH, Laufer N. The monitoring of ovulation induction using ultrasound and estrogen. Clin Obstet Gynecol 1984; 27:993–1002.
18. Rossavik IK, Gibbons WE. Variability of ovarian follicular growth in natural menstrual cycles. Fertil Steril 1985; 44:195–199.
19. Zegers-Hochschild F, Gomez Lira C, Parada M. A comparative study of the follicular growth profile in conception and nonconception cycles. Fertil Steril 1984; 41:244–247.
20. Baerwald AR, Walker RA, Pierson RA. Growth and regression rates of ovarian follicles during natural menstrual cycles and oral contraception cycles. In: Proceedings of the International Federation of Fertility Societies 18th World Congress on Fertility and Sterility, 2004. Abstracts:115.
21. Baerwald AR, Walker RA, Pierson RA. Growth rates of ovarian follicles during natural menstrual cycles and ovarian stimulation cycles. In: Proceedings of the 50th Annual Conference of the Canadian Fertility and Andrology Society, 2004:68.
22. Singh J, Adams GP, Pierson RA. Promise of new imaging technologies for assessing ovarian function. Anim Reprod Sci 2003; 78:371–399.
23. Sarty GE, Pierson RA. An application of Lacker's mathematical model for the prediction of ovarian response to superstimulation. Math Biosci 2005; 198:80–96.
24. Bukulmez O, Arici A. Assessment of ovarian reserve. Curr Opin Obstet Gynecol 2004; 16:231–237.
25. Broekmans FJ, Scheffer GJ, Bancsi LF. Ovarian reserve tests in infertility practice and normal fertile women. Maturitas 1998; 30:205–214.
26. Bukman A, Heineman MJ. Ovarian reserve testing and the use of prognostic models in patients with subfertility. Hum Reprod Update 2001; 7:581–590.
27. Erdem M, Erdem A, Gursoy R. Comparison of basal and clomiphene citrate induced FSH and inhibin B, ovarian volume and antral follicle counts as ovarian reserve tests and predictors of poor ovarian response in IVF. J Assist Reprod Genet 2004; 21:37–45.

28. Lass A. Assessment of ovarian reserve—is there a role for ovarian biopsy? Hum Reprod 2001; 16:1055–1057.
29. Syrop CH, Dawson JD, Husman KJ. Ovarian volume may predict assisted reproductive outcomes better than follicle stimulating hormone concentration on day 3. Hum Reprod 1999; 14:1752–1756.
30. Erdem A, Erdem M, Biberoglu K. Age-related changes in ovarian volume, antral follicle counts and basal FSH in women with normal reproductive health. J Reprod Med 2002; 47:835–839.
31. Bancsi LF, Broekmans FJ, Eijkemans MJ. Predictors of poor ovarian response in in vitro fertilization: a prospective study comparing basal markers of ovarian reserve. Fertil Steril 2002; 77:328–336.
32. Tufan E, Elter K, Durmusoglu F. Assessment of reproductive ageing patterns by hormonal and ultrasonographic ovarian reserve tests. Hum Reprod 2004; 19:2484–2489.
33. Lass A, Skull J, McVeigh E. Measurement of ovarian volume by transvaginal sonography before ovulation induction with human menopausal gonadotrophin for in vitro fertilization can predict poor response. Hum Reprod 1997; 12:294–297.
34. Durmusoglu F, Elter K, Yoruk P. Combining cycle day 7 follicle count with the basal antral follicle count improves the prediction of ovarian response. Fertil Steril 2004; 81:1073–1078.
35. Hendriks DJ, Mol BW, Bancsi LF. Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: a meta-analysis and comparison with basal follicle-stimulating hormone level. Fertil Steril 2005; 83:291–301.
36. Vladimkov IK, Tacheva DM, Kalinov KB. Prognostic value of some ovarian reserve tests in poor responders. Arch Gynecol Obstet 2005; 272:74–79.
37. Wallace WH, Kelsey TW. Ovarian reserve and reproductive age may be determined from measurement of ovarian volume by transvaginal sonography. Hum Reprod 2004; 19:1612–1617.
38. Pierson RA, Chizen DR, Olatunbosun OA. Ultrasonographic assessment of ovulation induction. In: Jaffe R, Pierson RA, Abramowicz JA, eds. Imaging in Infertility and Reproductive Endocrinology. Philadelphia: J.B. Lippincott, 1994:155–166.
39. Danninger B, Brunner M, Obruca A. Prediction of ovarian hyperstimulation syndrome by ultrasound volumetric assessment [corrected] of baseline ovarian volume prior to stimulation. Hum Reprod 1996; 11:1597–1599.
40. Orvieto R. Can we eliminate severe ovarian hyperstimulation syndrome? Hum Reprod 2005; 20:320–322.
41. Ben-Ami M, Perlitz Y, Haddad S. The effectiveness of spectral and color Doppler in predicting ovarian torsion: a prospective study. Eur J Obstet Gynecol Reprod Biol 2002; 104:64–66.
42. Auslender R, Lavie O, Kaufman Y. Coiling of the ovarian vessels: a color Doppler sign for adnexal torsion without strangulation. Ultrasound Obstet Gynecol 2002; 20:96–97.
43. Pierson RA, Adams GP. Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: strange bedfellows. Theriogenology 1995; 43:105–112.

44. Singh J, Pierson RA, Adams GP. Ultrasound image attributes of bovine ovarian follicles: endocrine and functional correlates. J Reprod Fertil 1988; 112:345–369.
45. Russell JB, DeCherney AH, Hobbins JC. A new transvaginal probe and biopsy guide for oocyte retrieval. Fertil Steril 1987; 47:350–352.
46. Cohen J, Debache C, Pez JP. Transvaginal sonographically controlled ovarian puncture for oocyte retrieval for in vitro fertilization. J In Vitro Fert Embryo Transf 1986; 3:309–313.
47. Dellenbach P, Nisand I, Moreau L. Transvaginal sonographically controlled follicle puncture for oocyte retrieval. Fertil Steril 1985; 44:656–662.
48. Hamberger L, Wikland M, Enk L. Laparoscopy versus ultrasound guided puncture for oocyte retrieval. Acta Eur Fertil 1986; 17:195–198.
49. Feichtinger W. Current technology of oocyte retrieval. Curr Opin Obstet Gynecol 1992; 4:697–701.
50. Gembruch U, Diedrich K, Welker B. Transvaginal sonographically guided oocyte retrieval for in vitro fertilization. Hum Reprod 1988; 3(suppl 2):59–63.
51. Feldberg D, Goldman JA, Ashkenazi J. Transvaginal oocyte retrieval controlled by vaginal probe for in vitro fertilization: a comparative study. J Ultrasound Med 1988; 7:339–343.
52. Schulman JD, Dotfinann AD, Jones SL. Outpatient in vitro fertilization using transvaginal ultrasound-guided oocyte retrieval. Obstet Gynecol 1987; 69: 665–668.
53. Wikland M, Enk L, Hammarberg K. Use of a vaginal transducer for oocyte retrieval in an IVF/ET program. J Clin Ultrasound 1987; 15:245–251.
54. Wikland M, Enk L, Hammarberg K. Oocyte retrieval under the guidance of a vaginal transducer. Ann NY Acad Sci 1988; 541:103–110.
55. Wiseman DA, Short WB, Pattinson HA. Oocyte retrieval in an in vitro fertilization–embryo transfer program: comparison of four methods. Radiology 1989; 173:99–102.
56. Diedrich K, Van det Ven H, Al-Hasani S. Oocyte retrieval for in vitro fertilization. Ann Biol Clin 1987; 45:351–357.
57. Padilla SL, Butler WJ, Boldt JP. Transurethral ultrasound-guided oocyte retrieval for in vitro fertilization. Am J Obstet Gynecol 1987; 157:622–626.
58. Parsons J, Riddle A, Booker M. Oocyte retrieval for in vitro fertilisation by ultrasonically guided needle aspiration via the urethra. Lancet 1985; 1(8437):1076–1077.
59. Kemeter P, Feichtinger W. Trans-vaginal oocyte retrieval using a trans-vaginal sector scan probe combined with an automated puncture device. Hum Reprod 1986; 1:21–24.
60. Schulman JD, Dorfinann A, Jones S. Outpatient in vitro fertilization using transvaginal oocyte retrieval and local anesthesia. N Engl J Med 1985; 312(25):1639.
61. Sterzik K, Jonatha W, Keckstein G. Ultrasonically guided follicle aspiration for oocyte retrieval in an in vitro fertilization program: further simplification. Int J Gynaecol Obstet 1987; 25:309–314.
62. Yuzpe AA, Brown SE, Casper RF. Transvaginal, ultrasound-guided oocyte retrieval for in vitro fertilization. J Reprod Med 1989; 34:937–942.
63. Ragni G, Lombroso GC, De Lauretis L. Echoguided transvaginal oocyte retrieval: effective and easy to learn technique. Acta Eur Fertil 1991; 22:89–90.

64. Kingsland CR, Taylor CT, Aziz N. Is follicular flushing necessary for oocyte retrieval? A randomized trial. Hum Reprod 1991; 6:382–383.
65. Knight DC, Tyler JP, Driscoll GL. Follicular flushing at oocyte retrieval: a reappraisal. Aust NZ J Obstet Gynaecol 2001; 41:210–213.
66. Bustillo M. Unsuccessful oocyte retrieval: technical artefact or genuine 'empty follicle syndrome'? Reprod Biomed Online 2004; 8:59–67.
67. Barak Y, Lessing JB, Amit A. The development of an efficient ambulatory in vitro fertilization (IVF) and embryo transfer (ET) program using ultrasonically guided oocyte retrieval. Acta Obstet Gynecol Scand 1988; 67:585–588.
68. Tureck RW, Garcia CR, Blasco L. Perioperative complications arising after transvaginal oocyte retrieval. Obstet Gynecol 1993; 81:590–593.
69. Christiaens F, Janssenswillen C, Van Steirteghem AC. Comparison of assisted reproductive technology performance after oocyte retrieval under general anaesthesia (propofol) versus paracervical local anaesthetic block: a case-controlled study. Hum Reprod 1998; 13:2456–2460.
70. El-Shawarby S, Margara R, Trew G. A review of complications following transvaginal oocyte retrieval for in vitro fertilization. Hum Fertil 2004; 7:127–133.
71. Ng EH, Miao B, Ho PC. Anxiolytic premedication reduces preoperative anxiety and pain during oocyte retrieval: a randomized double-blinded placebo-controlled trial. Hum Reprod 2002; 17:1233–1238.
72. Tummon I, Newton C, Lee C. Lidocaine vaginal gel versus lidocaine paracervical block for analgesia during oocyte retrieval. Hum Reprod 2004; 19:1116–1120.
73. Fleischer AC, Herbert CM, Sacks GA. Sonography of the endometrium during conception and nonconception cycles of in vitro fertilization and embryo transfer. Fertil Steril 1986; 46:442–447.
74. Gonen Y, Casper RF, Jacobson W. Endometrial thickness and growth during ovarian stimulation: a possible predictor of implantation in in vitro fertilization. Fertil Steril 1989; 52:446–450.
75. Dickey RP, Olar TT, Curole DN. Endometrial pattern and thickness associated with pregnancy outcome after assisted reproduction technologies. Hum Reprod 1992; 7:418–421.
76. Dickey RP, Olar TT, Taylor SN. Relationship of endometrial thickness and pattern to fecundity in ovulation induction cycles: effect of clomiphene citrate alone and with human menopausal gonadotropin. Fertil Steril 1993; 59:756–760.
77. Ueno J, Oehninger S, Brzyski RG. Ultrasonographic appearance of the endometrium in natural and stimulated in vitro fertilization cycles and its correlation with outcome. Hum Reprod 1991; 6:901–904.
78. Khalifa E, Brzyski RG, Oehninger S. Sonographic appearance of the endometrium: the predictive value for the outcome of in vitro fertilization in stimulated cycles. Hum Reprod 1992; 7:677–680.
79. Abdalla HI, Brooks AA, Johnson MR. Endometrial thickness: a predictor of implantation in ovum recipients? Hum Reprod 1994; 9:363–365.
80. Coulam CB, Bustulo M, Soenksen DM. Ultrasonographic predictors of implantation after assisted reproduction. Fertil Steril 1994; 62:1004–1010.
81. Noyes N, Iiu HC, Sultan K. Endometrial thickness appears to be a significant factor in embryo implantation in in vitro fertilization. Hum Reprod 1995; 10:919–922.

82. Potlog-Nahari C, Catherino WH, McKeeby JL. A suboptimal endometrial pattern is associated with a reduced likelihood of pregnancy after a day 5 embryo transfer. Fertil Steril 2005; 83:235–237.
83. Graf MJ, Reyniak JV, Battle-Mutter P. Histologic evaluation of the luteal phase in women following follicle aspiration for oocyte retrieval. Fertil Steril 1988; 49:616–619.
84. Sterzik K, Grab D, Schneider V. Lack of correlation between ultrasonography and histologic staging of the endometrium in in vitro fertilization (IVF) patients. Ultrasound Med Biol 1997; 23:165–170.
85. Csemiczky G, Wramsby H, Johannisson E. Endometrial evaluation is not predictive for in vitro fertilization treatment. J Assist Reprod Genet 1999; 16:113–116.
86. Sundstrom P. Establishment of a successful pregnancy following in vitro fertilization with an endometrial thickness of no more than 4 mm. Hum Reprod 1998; 13:1550–1552.
87. Zhang X, Chen CH, Confino E. Increased endometrial thickness is associated with improved treatment outcome for selected patients undergoing in vitro fertilization–embryo transfer. Fertil Steril 2005; 83:336–340.
88. Chien LW, Au HK, Xiao J. Fluid accumulation within the uterine cavity reduces pregnancy rates in women undergoing IVF. Hum Reprod 2002; 17:351–356.
89. Zagzebski J. Essentials of Ultrasound Physics. New York: C.V. Mosby, 1996:220.
90. Sterzik K, Grab D, Sasse V. Doppler sonographic findings and their correlation with implantation in an in vitro fertilization program. Fertil Steril 1989; 52: 825–828.
91. Steer CV, Campbell S, Tan SL. The use of transvaginal color flow imaging after in vitro fertilization to identify optimum uterine conditions before embryo transfer. Fertil Steril 1992; 57:372–376.
92. Serafini P, Batzofin J, Nelson J. Sonographic uterine predictors of pregnancy in women undergoing ovulation induction for assisted reproductive treatments. Fertil Steril 1994; 62:815–822.
93. Tekay A, Martikainen H, Jouppila P. Blood flow changes in uterine and ovarian vasculature, and predictive value of transvaginal pulsed colour Doppler ultrasonography in an in vitro fertilization programme. Hum Reprod 1995; 10:688–693.
94. Bassil S, Magritte JP, Roth J. Uterine vascularity during stimulation and its correlation with implantation in in vitro fertilization. Hum Reprod 1995; 10: 1497–1501.
95. Cacciatore B, Simbetg N, Fusaro P. Transvaginal Doppler study of uterine artery blood flow in in vitro fertilization–embryo transfer cycles. Fertil Steril 1996; 66:130–134.
96. Aytoz A, Ubaldi F, Tournaye H. The predictive value of uterine artery blood flow measurements for uterine receptivity in an intracytoplasmic sperm injection program. Fertil Steril 1997; 68:935–937.
97. Friedler S, Schenker JG, Herman A. The role of ultrasonography in the evaluation of endometrial receptivity following assisted reproductive treatments: a critical review. Hum Reprod Update 1996; 2:323–335.
98. Yokota A, Nakai A, Oya A. Changes in uterine and ovarian arterial impedance during the periovulatory period in conception and nonconception cycles. J Obstet Gynaecol Res 2000; 26:435–440.

99. Zaidi J, Campbell S, Pittrof R. Endometrial thickness, morphology, vascular penetration and velocimetry in predicting implantation in an in vitro fertilization program. Ultrasound Obstet Gynecol 1995; 6:191–198.
100. Yang JH, Wu MY, Chen CD. Association of endometrial blood flow as determined by a modified colour Doppler technique with subsequent outcome of in vitro fertilization. Hum Reprod 1999; 14:1606–1610.
101. Schild RL, Knobloch C, Dorn C. Endometrial receptivity in an in vitro fertilization program as assessed by spiral artery blood flow, endometrial thickness, endometrial volume, and uterine artery blood flow. Fertil Steril 2001; 75: 361–366.
102. Kupesic S, Bekavac I, Bjelos D. Assessment of endometrial receptivity by transvaginal color Doppler and three-dimensional power Doppler ultrasonography in patients undergoing in vitro fertilization procedures. J Ultrasound Med 2001; 20:125–134.
103. Applebaum M. The uterine biophysical profile. Ultrasound Obstet Gynecol 1995; 5:67–68.
104. Salle B, Bied-Damon V, Benchaib M. Preliminary report of an ultrasonography and colour Doppler uterine score to predict uterine receptivity in an in vitro fertilization programme. Hum Reprod 1998; 13:1669–1673.
105. Pierson RA. Three-dimensional ultrasonography of the embryo and fetus. In: Jaffe R, Bui TH, eds. Fetal and Neonatal Ultrasonography. New York: Parthenon Publishing Group, 1999:317–326.
106. Raga F, Bonilk-Musoles F, Casan EM. Assessment of endometrial volume by three-dimensional ultrasound prior to embryo transfer: clues to endometrial receptivity. Hum Reprod 1999; 14:2851–2854.
107. Schild RL, Indefrei D, Eschweiler S. Three-dimensional endometrial volume calculation and pregnancy rate in an in vitro fertilization programme. Hum Reprod 1999; 14:1255–1258.
108. Schild RL, Holthaus S, d'Alquen J. Quantitative assessment of subendometrial blood flow by three-dimensional-ultrasound is an important predictive factor of implantation in an in vitro fertilization programme. Hum Reprod 2000; 15: 89–94.
109. Wu HM, Chiang CH, Huang HY. Detection of the subendometrial vascularization flow index by three-dimensional ultrasound may be useful for predicting the pregnancy rate for patients undergoing in vitro fertilization–embryo transfer. Fertil Steril 2003; 79:507–511.
110. Martinez-Gaudio M, Yoshida T, Bengtsson LP. Propagated and nonpropagated myometrial contractions in normal menstrual cycles. Am J Obstet Gynecol 1973; 115:107–111.
111. de Vries K, Lyons EA, Ballard G. Contractions of the inner third of the myometrium. Am J Obstet Gynecol 1990; 162:679–682.
112. Lyons EA, Taylor PJ, Zheng XH. Characterization of subendometrial myometrial contractions throughout the menstrual cycle in normal fertile women. Fertil Steril 1991; 55:771–774.
113. Ijland MM, Evers JL, Dunselman GA. Endometrial wavelike movements during the menstrual cycle. Fertil Steril 1996; 65:746–749.

114. Fanchin R, Righini C, Ayoubi JM. New look at endometrial echogenicity: objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization–embryo transfer. Fertil Steril 2000; 74:274–281.
115. Ijland MM, Evers JL, Hoogland HJ. Velocity of endometrial wavelike activity in spontaneous cycles. Fertil Steril 1997; 68:72–75.
116. van Gestel I, Ijland MM, Hoogland HJ. Endometrial wave-like activity in the non-pregnant uterus. Hum Reprod Update 2003; 9:131–138.
117. Lesny P, Killick SR. The junctional zone of the uterus and its contractions. BJOG 2004; 111:1182–1189.
118. Lesny P, Killick SR, Tetlow RL. Uterine junctional zone contractions during assisted reproduction cycles. Hum Reprod Update 1998; 4:440–445.
119. Fanchin R, Righini C, Ayoubi JM. Uterine contractions at the time of embryo transfer: a hindrance to implantation?. Contracept Fertil Sex 1998; 26:498–505.
120. Fanchin R. Assessing uterine receptivity in 2001: ultrasonographic glances at the new millennium. Ann NY Acad Sci 2001; 943:185–202.
121. Fanchin R, Righini C, Olivennes F. Uterine contractions at the time of embryo transfer alter pregnancy rates after in vitro fertilization. Hum Reprod 1998; 13:1968–1974.
122. Vlaisavljevic V, Reljic M, Gavric Lovrec V. Subendometrial contractility is not predictive for in vitro fertilization (IVF) outcome. Ultrasound Obstet Gynecol 2001; 17:239–244.
123. Fanchin R, Righini C, de Ziegler D. Effects of vaginal progesterone administration on uterine contractility at the time of embryo transfer. Fertil Steril 2001; 75:1136–1140.
124. Baruffi R, et al. Effects of vaginal progesterone administration starting on the day of oocyte retrieval on pregnancy rates. J Assist Reprod Genet 2003; 20(12):517–520.
125. Bulletti C, de Ziegler D. Uterine contractility and embryo implantation. Curr Opin Obstet Gynecol 2005; 17:265–276.
126. Fanchin R, Ayoubi JM, Righini C. Uterine contractility decreases at the time of blastocyst transfers. Hum Reprod 2001; 16:1115–1119.
127. Strickler RC, Christianson C, Crane JP. Ultrasound guidance for human embryo transfer. Fertil Steril 1985; 43:54–61.
128. Leong M, Leung C, Tucker M. Ultrasound-assisted embryo transfer. J In Vitro Fert Embryo Transf 1986; 3:383–385.
129. al-Shawaf T, Dave R, Harper J. Transfer of embryos into the uterus: how much do technical factors affect pregnancy rates?. J Assist Reprod Genet 1993; 10:31–36.
130. al-Shawaf T, Yang D, al-Magid Y. Ultrasonic monitoring during replacement of frozen/thawed embryos in natural and hormone replacement cycles. Hum Reprod 1993; 8:2068–2074.
131. Kan AK, Abdalla HI, Gafar AH. Embryo transfer: ultrasound-guided versus clinical touch. Hum Reprod 1999; 14:1259–1261.
132. Coroleu B, Carreras O, Veiga A. Embryo transfer under ultrasound guidance improves pregnancy rates after in vitro fertilization. Hum Reprod 2000; 15:616–620.
133. Rijnders RJ, Mol BW, Broekmans FJ. Use of ultrasound guidance during embryo transfer. Hum Reprod 2000; 15:2449.

134. Anderson RE, Nugent NL, Gregg AT. Transvaginal ultrasound-guided embryo transfer improves outcome in patients with previous failed in vitro fertilization cycles. Fertil Steril 2002; 77:769–775.
135. Biervliet FP, Lesny P, Maguiness SD. Transmyometrial embryo transfer and junctional zone contractions. Hum Reprod 2002; 17:347–350.
136. Baba K, Ishihara O, Hayashi N. Three-dimensional ultrasound in embryo transfer. Ultrasound Obstet Gynecol 2000; 16:372–373.
137. de Camargo Martins AM, Baruffi RL, Mauri AL. Ultrasound guidance is not necessary during easy embryo transfers. J Assist Reprod Genet 2004; 21:421–425.
138. Kojima K, Nomiyama M, Kumamoto T. Transvaginal ultrasound-guided embryo transfer improves pregnancy and implantation rates after IVF. Hum Reprod 2001; 16:2578–2582.
139. Sieck UV, Jaroudi KA, Hollanders JM. Ultrasound guided embryo transfer does not prevent ectopic pregnancies after in vitro fertilization. Hum Reprod 1997; 12:2081–2082.
140. Letterie GS. Three-dimensional ultrasound-guided embryo transfer: a preliminary study. Am J Obstet Gynecol 2005; 192:1983–1987; discussion 1987–1988.
141. Tang OS, Ng EH, So WW. Ultrasound-guided embryo transfer: a prospective randomized controlled trial. Hum Reprod 2001; 16:2310–2315.
142. Matorras R, Urquijo E, Mendoza R. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. Hum Reprod 2002; 17:1762–1766.
143. Letterie GS, Marshall L, Angle M. A new coaxial catheter system with an echodense tip for ultrasonographically guided embryo transfer. Fertil Steril 1999; 72:266–268.
144. Mansour RT, Aboulghar MA. Optimizing the embryo transfer technique. Hum Reprod 2002; 17:1149–1153.
145. Hale L. Embryo transfer: how to ensure correct placement in utero. Reprod Fertil Dev 2001; 13:95–98.
146. Schoolcraft WB, Surrey ES, Gardner DK. Embryo transfer: techniques and variables affecting success. Fertil Steril 2001; 76:863–870.
147. Coroleu B, Batri PN, Carreras O. The influence of the depth of embryo replacement into the uterine cavity on implantation rates after IVF: a controlled, ultrasound-guided study. Hum Reprod 2002; 17:341–346.
148. Pope CS, Cook EK, Arny M. Influence of embryo transfer depth on in vitro fertilization and embryo transfer outcomes. Fertil Steril 2004; 81:51–58.
149. Wood EG, Batzer FR, Go KJ. Ultrasound-guided soft catheter embryo transfers will improve pregnancy rates in in vitro fertilization. Hum Reprod 2000; 15:107–112.
150. Lindheim SR, Cohen MA, Sauer MV. Ultrasound guided embryo transfer significantly improves pregnancy rates in women undergoing oocyte donation. Int J Gynaecol Obstet 1999; 66:281–284.
151. Prapas Y, Prapas N, Hatziparasidou A. Ultrasound-guided embryo transfer maximizes the IVF results on day 3 and day 4 embryo transfer but has no impact on day 5. Hum Reprod 2001; 16:1904–1908.
152. Coroleu B, Barri PN, Carreras O. The usefulness of ultrasound guidance in frozen-thawed embryo transfer: a prospective randomized clinical trial. Hum Reprod 2002; 17:2885–2890.

153. Garcia-Velasco JA, Isaza V, Martinez-Salazar J. Transabdominal ultrasound-guided embryo transfer does not increase pregnancy rates in oocyte recipients. Fertil Steril 2002; 78:534–539.
154. Sallam HN. Should embryo transfer always be performed under ultrasound guidance? Ultrasound Obstet Gynecol 2004; 24:383–386.
155. Shamonki MI, Schattman GL, Spandorfer SD. Ultrasound-guided trial transfer may be beneficial in preparation for an IVF cycle. Hum Reprod 2005; 20:2844–2849.
156. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. Fertil Steril 2003; 80:1037–1041.
157. Sallam HN, Sadek SS. Ultrasound-guided embryo transfer: a meta-analysis of randomized controlled trials. Fertil Steril 2003; 80:1042–1046.
158. Li R, Lu L, Hao G. Abdominal ultrasound-guided embryo transfer improves clinical pregnancy rates after in vitro fertilization: experiences from 330 clinical investigations. J Assist Reprod Genet 2005; 22:3–8.

7

Oocyte Retrieval

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INTRODUCTION

Laparoscopy

Before the era of clinical in vitro fertilization (IVF), oocytes were acquired by laparotomy (1). Aside from the escalating scientific interest in fertilizing and culturing human oocytes in vitro, the development of laparoscopic surgery also facilitated the use of IVF in treatment of infertile couples. At the end of 1960, the laparoscopic technique made it possible to retrieve human oocytes quite simply for both research and clinical purposes. The technique played a key role in the start of collaboration between Edwards, the scientist with the knowledge of how to fertilize and culture human oocytes in vitro, and Steptoe, a clinician mastering a technique that could be used to harvest oocytes in women with tubal infertility (2). Subsequently, laparoscopy became the technique of choice for oocyte aspiration during the first 10 years of this clinical IVF era. Different technical aspects surrounding this technique were published at that time by those groups involved in the early clinical practice of IVF (3–5). Retrieving oocytes under the guidance of a laparoscope was a fairly simple technique with a high oocyte recovery rate. However, general anesthesia was routinely needed and only in certain patients was it performed with local anesthesia (6). Furthermore, laparoscopy exposes healthy women to risks that are not negligible and it is rather an expensive method. In order for IVF to evolve into a procedure that could

be afforded by many women, there was a need to find a technique for oocyte retrieval that could be performed as an outpatient surgical procedure. Even in those cases when laparoscopy was performed using local anesthesia, it was still quite a technically demanding technique using expensive instruments and unsuitable to performing multiple oocyte collections on the same day (6). With the introduction of ultrasound in monitoring follicular maturation and the knowledge that ultrasound could be used for guiding puncturing procedures, it is not surprising that ultrasound-guided follicle aspiration became a very interesting alternative technique.

Ultrasound

Clinical IVF started at the time when ultrasound imaging in obstetrics and gynecology developed rapidly. This was due to a rapid technical development within diagnostic ultrasound. Ultrasound was applied early for ovarian imaging and in some early studies it was elegantly shown that the technique could be used for monitoring follicular maturation (7,8). Furthermore, one of the Danish pioneers within diagnostic ultrasound proved earlier that the technique could be used safely and accurately for guidance of percutaneous puncture of cystic as well as homogenous tumors within different organs of the body (9). With the experience of ultrasound-guided puncture developed by Holm et al., it is not surprising that another Danish group lead by Lenz (10) demonstrated the first successful oocyte collection under the guidance of ultrasound. Parallel to Lenz et al., our own group in the beginning of 1980 started to use a similar technique for ultrasound-guided follicle aspiration (11). The technique used at that time utilized abdominal ultrasound scanning and transabdominal, transvesical puncture of follicles. This technique had to be performed in light general anesthesia with a full urinary bladder (12). Although it was not as technically demanding as laparoscopy, it was still preferable to perform in general anesthesia and as such the advantages in comparison to laparoscopy were not widely appreciated. However, many IVF groups did adopt the technique and some tried new approaches that could be used with local anesthesia. One such interesting development was the introduction of the puncturing needle through a urinary catheter to the urinary bladder. By scanning abdominally, the needle could be directed to the follicles (13).

In 1983, our group started to work on vaginal ultrasound scanning. At that time, we used small abdominal mechanical sector transducers that after some modification could be used for vaginal scanning. However, those probes were not ideal for puncturing transvaginally. We, therefore, started a collaboration with the Danish ultrasound company Bryel and Kjaer. They had developed a probe for cranial scanning during neurosurgical procedures. After minor modifications of this probe, it could be used as a vaginal transducer. Ovaries could now easily be scanned without the full bladder

technique, and oocyte aspiration could be performed as an outpatient procedure with local anesthesia (14). Manufacturers of diagnostic ultrasound very soon realized the potential of vaginal sonography for gynecological diagnostic and interventional procedures and consequently started to produce vaginal probes. It did not take long for other IVF groups to realize the potential of this new technique. One group that begun early was Dellenbach et al. in France (15,16) and they published their initial experiences already in 1984. Another group that contributed early on to the development of this technique were Feichtinger and Kemeter (17).

Transvaginal ultrasound-guided oocyte retrieval (TVOR) has since then become the gold standard for oocyte aspiration in assisted reproductive technology (ART). Even though no large prospective-controlled, randomized trial comparing laparoscopic-guided and transvaginal-guided oocyte retrieval has ever been performed, the latter has become the method of choice. The reason for this is probably that TVOR is such a simple method and does in the majority of patients result in enough oocytes to guarantee a good chance of becoming pregnant.

The technique has, over the years, in many ways been improved. The ultrasound equipment has become more sophisticated, resulting in extremely refined images as compared to the equipment used more than 20 years ago. This has meant a lot for the safety of this technique and the ease with which it can be used.

TECHNICAL ASPECTS

Equipment

Today most ultrasound equipment with a vaginal transducer can be used for safe and accurate puncture of follicles via the vaginal route. It is, however, important to chose a fairly long (total length 40–50 cm) transducer, which makes it easy to handle during the scanning and puncture procedure. A frequency of 5–7 MHz gives a sufficient penetration depth and enough resolution for accurate scanning of the lower pelvis. The transducer should have a shape that is easy to put into a slim sterile cover or a finger of a sterile surgical glove. The needle guide should be easy to attach to the transducer when it has been placed in a sterile cover (Fig. 1).

There are today many different needles that are specially designed for oocyte aspiration. Most companies, like Cook Ltd., Swemed International Lab, and Wallas Ltd., which have specialized in equipment for IVF, offer different types of needles for oocyte retrievals. Our opinion after nearly 20 years of experience with TVOR and having tested most of the available needles on the market is that the sharpness of the needle is the most important factor. A sharp needle means less pain if the puncture is performed in analgesia. Furthermore, it is important that the surface of needle tip has

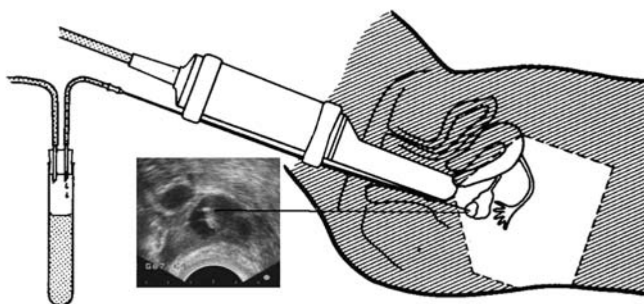

Figure 1 Schematic illustration of transvaginal ultrasound-guided oocyte retrieval. The needle is inserted through the needle guide that is mounted on the vaginal transducer. The ultrasound image shows a punctured follicle, and the needle tip is presented as a white echo inside the follicle.

some kind of preparation that will increase the ultrasound echo, making it easier to identify the position of the needle tip. Most needles used for ultrasound-guided puncture do have such a preparation. The diameter of the needle is important for two reasons. A thin needle 18–20 gage means less pain when the analgesia only is used. The other is that a needle with too small inner diameter may be harmful to the oocyte cumulus complex (18). As long as the inner diameter of the needle is 0.8–1 mm, it seems as if the oocyte cumulus complex is unaffected, provided that the aspiration pressure is <120 mmHg. A needle too thin often causes problems by deviating away from the puncturing line, particularly if the ovary is situated high up in the pelvis. A nicely shaped fingertip handle on the distal end of the needle is preferable because it makes it possible to puncture with good clinical touch.

To increase the recovery, it was shown earlier that Teflon tubing between the needle and the sampling tube was important. Commercially available follicle aspiration needles do have such tubing as well as a sampling tube. Today, manufactures provide sterile and mouse embryo tested follicle aspiration sets including needle, tubing, and sampling tubes. The set is ready to use and only needs to be connected to the suction pump. The set is made for single use and thereby guarantees sterility and non-toxicity to the oocyte. Over the last 15 years, our group has used such follicle aspiration sets and found them to be very convenient. In our opinion, an 18 gage needle (outer diameter) thin walled with an inner diameter of 20 gage is ideal.

Suction of follicular fluid can be performed either with a syringe or a suction pump creating the negative pressure needed. It was shown earlier that a negative pressure of 90–120 mmHg seems to be optimal for a good recovery and exerts no harm on the oocyte cumulus complex when aspirating mature follicles. However, aspirating immature oocytes from follicles of

5 mm diameter with very small volume needs much less pressure, 40–60 mmHg (19,20). Creating a negative pressure by means of a suction pump where the pressure can be controlled in a standardized manner is probably the safest and the best way. Today there are several such suction pumps available specifically manufactured for oocyte aspiration. In the early days, aspiration of follicular fluid was often performed by means of a syringe connected to the puncturing needle. This is a risky alternative because it is difficult to control the negative pressure and it was earlier shown that the effect of a high negative pressure in the system might cause damage to the oocyte cumulus complex (21).

In conclusion, one should use a ready-to-use follicle aspiration set and connect it to a calibrated suction pump using a negative pressure of 100 mmHg for retrieval of mature oocytes and 50 mmHg for immature oocytes.

Aspiration technique

The actual TVOR technique has been described extensively over the years and in principle has not changed very much (Fig. 1) (22,23). I will, therefore, in this part only focus on the following: anesthesia/analgesia; flushing follicles or not, and complications.

The first two topics are interesting because almost all IVF clinics have their own methods of management. It is also very important to discuss updates related to complications within a technique. The longer a technique has been in use, the more information we will acquire about its safety.

Anesthesia or Analgesia

Already from the beginning our group performed TVOR combined with conscious sedation. Our reason was driven by the belief that if TVOR could be accepted by the patients and performed under local anesthesia, it would bring down the cost of the whole IVF treatment.

Oocyte retrieval is supposed to be physically the most painful part of the IVF procedure, and various methods of analgesia have been tried over the years. Many groups therefore use general anesthesia. In our opinion, one of the leading advantages of transvaginal follicle puncture is then lost. A good analgesic method for oocyte retrieval has to give a satisfactory pain relief with rapid onset, rapid recovery, and ease of administration and monitoring. It is also important that it is safe and has no toxic effect on the oocytes.

Conscious sedation appears to be the most commonly used method of pain relief for oocyte retrieval in the United Kingdom and in the United States (24,25).

Randomized, controlled trials (RTC) suggest that pain relief is superior when a paracervical block (PCB) is used combined with sedation

as compared to sedation alone (26,27). It has also been shown that patients who received only a PCB during egg collection experienced 2.5 times higher levels of vaginal and abdominal pain as compared to those who received both PCB and conscious sedation (28). With the PCB, the local anesthetic is usually deposited in four locations around the cervix in the vaginal mucosa. Accordingly, today we use in total 100 mg lidocaine (10 ml of 1% lidocaine, Xylocaine™ 10 mg/ml, AstraZeneca Sverige AB, Södertälje, Sweden) injected at four points around the cervix and alfentanil 0.5 mg IV. If needed, a supplementary 0.25 mg alfentanil (Rapifen 0.5 mg/ml; Janssen-Cilag AB, Sollentuna, Sweden) is given once or twice during the procedure. With this combination, 99.5% of our oocyte aspirations are performed. The majority of our patients seem to have sufficient pain relief (29).

An interesting method for pain relief during oocyte aspiration is electro-acupuncture. The reason for exploring this method arose from an earlier study from our group where we tried to evaluate a possible positive effect of electro-acupuncture on ovarian blood flow (30). This study led us to also explore the possible positive effect on pain during oocyte aspiration (29). A recently published study comparing electro-acupuncture and conventional medical analgesia during oocyte aspiration showed, however, that no method seems to be superior to another (31). Hence, our experience is that electro-acupuncture can in many patients be a good alternative for pain relief during oocyte aspiration (32).

Flushing Follicles or Not

Flushing follicles or not has been debated for years. The rationale is that flushing of the follicle offers an advantage to the patient, with a larger number of oocytes being collected and therefore a higher potential for pregnancy. Kingsland et al. were the first to perform a randomized trial. They demonstrated that when performing transvaginal ultrasonically guided oocyte recovery, there were no significant differences in number of oocytes retrieved, fertilization rate, or pregnancy rate between those where flushing had been used as compared to no flushing. However, they could show that the operating time was significantly shortened in the non-flushing group (33). Similar results were later demonstrated in another prospective randomized study (34). In a recent publication, a study by Bagtharia and Haloob found that 40% of the oocytes were retrieved in the primary aspirate, whereas up to 82% of oocytes were retrieved with two flushes and 97% of oocytes were retrieved in up to four flushes. Based on this study, they concluded that the optimum number of follicular flushings was four times (35). It could be argued that in this particular study it is not known if the oocytes were trapped in the needle or the tubing after the first aspiration. In our IVF program, we do not flush follicles and have had a recovery rate of 70% per punctured follicle over the last 10 years. Based on our own experience and of

published prospective randomized studies, in our opinion routine flushing would seem superfluous in ART.

COMPLICATIONS

In this part, I will only deal with complications related to TVOR because this is the gold standard today for oocyte collection in ART. Complications related to laparoscopic procedures are dealt with elsewhere.

Despite all the advantages with TVOR during IVF treatment, the aspiration needle may injure pelvic organs and structures leading to serious complications. The most common complications are hemorrhage, trauma, and injury of pelvic structures and pelvic infection. Other complications described include adnexal torsion, rupture of endometriotic cysts, and even vertebral osteomyelit (36).

Bleeding from the vaginal vault is the most common consequence and has been reported to occur between 1.4% and 18.4% (37). However, such a bleeding generally ceases spontaneously at the end of the procedure. Sometimes, the bleeding site needs to be identified and application of a sponge is necessary.

Injury to intraperitoneal or retroperitoneal pelvic blood vessels and subsequent bleeding have been reported to occur from 0% to 1.3% (38–40). Retroperitoneal bleeding can be difficult to diagnose due to the absence of free fluid in the pouch of Douglas and can be present several hours after oocyte pickup (41).

Acute severe intraabdominal bleeding is often detected by symptoms immediately after the OPU. Such symptoms are weakness, dizziness, dyspnea, abdominal pain, tachycardia, and low blood pressure typical for any severe bleeding. In such cases, early hemodynamic monitoring with serial measurement of hemoglobin concentrations should be performed. A drop in hemoglobin indicates intraabdominal bleeding and should be regarded as such until the opposite is proved. With the wide variation in TVOR and how it is performed today, one has to be aware that intraabdominal bleeding can always occur and any sign indicating bleeding should be taken seriously.

Pelvic infections after TVOR have been reported to occur between 0.2% and 0.5% (40,42). Symptoms such as lower abdominal pain more than a week after TVOR, dysuria, and fever should always make one suspicious of a pelvic infection. If endometrioma has been punctured during TVOR, there is always a risk for an infection. There are reports about tubo-ovarian or abscess after OPU (42). Owing to the risk for pelvic infections after OPU, it has been debated if prophylactic antibiotics should be given. However, even though the role of prophylactic antibiotics is controversial, they should be considered in the presence of risk factors (43). Our group does not use

any prophylactic antibiotics. Over the years, we have had four cases of pelvic abscess in almost 12,500 OPU. Of course, the risk of pelvic infection is related to many things such as if the patient is at risk for PID or not. Another important factor is the type of vaginal cleaning performed before the OPU. Over the years, we have only used sterile saline for washing of the vagina before OPU and still have had very few proven pelvic infections. However, one has to be aware of the risk of infection and if the patient presents herself with symptoms before embryo transfer, it is recommended to freeze the embryos for later transfer when the infection has been treated.

CONCLUSION

For more than 20 years, oocyte aspiration has been performed under the guidance of transvaginal ultrasound. The technique has proved to be a simple and safe procedure that is well accepted by the patients even if it is performed under local anesthesia with conscious sedation. It can thus be performed as an outpatient procedure. Since it was introduced and till today the technique has been refined in many ways and is regarded as the gold standard for oocyte pickup. The simplification that transvaginal ultrasound-guided oocyte retrieval has meant for the IVF procedure has probably given more patients access to ART.

REFERENCES

1. Edwards RG. Maturation in vitro of human ovarian oocytes. Lancet 1965; 2:926.
2. Steptoe PC. Laparoscopy and ovulation. Lancet 1968; 2:913.
3. Lopata A, Johnston IW, Leeton JF, et al. Collection of human oocytes at laparoscopy and laparotomy. Fertil Steril 1974; 25:1030.
4. Renou P, Traunson AO, Wood C, et al. The collection of human oocytes for in vitro fertilization. An instrument for maximising oocyte recovery rate. Fertil Steril 1981; 35:409.
5. Jones HW, Acosta AA, Garcia J. A technique for the aspiration of oocytes from human ovarian follicles. Fertil Steril 1982; 37:26.
6. Belaisch-Allart JC, Hazout A, Guillet-Rosso F, et al. Various techniques for oocyte recovery in an in vitro fertilization and embryo transfer program. J In Vitro Fert Embryo Transf 1985; 2:99.
7. Kratochwil A, Urban G, Friedrich F. Ultrasonic tomography of the ovaries. Ann Chir Gynaecol Fenn 1972; 61:211.
8. Hackeloer BJ, Flemming R, Robionsson HP, et al. Correlation of ultrasonic and endocrinologic assessment of human follicular development. Am J Obstet Gynecol 1979; 135:122.
9. Holm HH, Kristensen JK, Rasmussen HP, et al. Ultrasound as a guide in percutaneous puncture technique. Ultrasonics 1972; 10:83.

10. Lenz S, Lauritsen JG, Kjellow M. Collection of human oocytes for in vitro fertilization by ultrasonically guided puncture. Lancet 1981; 1:1163.
11. Hamberger L, Wikland M, Nilsson L. Methods for aspiration of human oocytes by various techniques. Acta Med Rom 1982; 20:370.
12. Wikland M, Nilsson L, Hansson R, et al. Collection of human oocytes by the use of sonography. Fertil Steril 1983; 39:603.
13. Parsons J, Riddell A, Booker M, et al. Oocyte retrieval for in-vitro fertilisation by ultrasonically guided needle aspiration via the urethra. Lancet 1985; 1:1076..
14. Wikland M, Enk L, Hamberger L. Transvesical and transvaginal approaches for the aspiration of follicles by use of ultrasound. Ann NY Acad Sci 1985; 442:182.
15. Dellenbach P, Nissand J, Morean L, et al. Transvaginal, sonographically controlled ovarian follicle puncture for egg retrieval. Lancet 1984; 1:1467.
16. Dellenbach P, Nissand J, Morean L, et al. Transvaginal sonographically controlled follicle puncture for oocyte retrieval. Fertil Steril 1985; 44:656.
17. Feichtinger W, Kemeter P. Transvaginal sector scan sonography for needle guided transvaginal follicle aspiration and other applications in gynecologic routine and research. Fertil Steril 1986; 45:722.
18. Awonuga A, Waterstone J, Ovesanya O, et al. A prospective randomized study comparing needles of different diameters for transvaginal ultrasound-directed follicle aspiration. Fertil Steril 1996; 65:109.
19. Mikkelsen AL, Smith S, Lindenberg S. Possible factors affecting the development of oocytes in in vitro maturation. Hum Reprod 2000; 15(suppl 5):11.
20. Papanikolaou EG, Platteau P, Albano C, et al. Immature oocyte in-vitro maturation: clinical aspects. Reprod Biomed Online 2005; 10:587.
21. Choen J, Avery S, Campbell S. Follicular aspiration using a syringe suction system may damage zona pellucid. J In Vitro Fertil Embryo Transf 1986; 4:224.
22. Wikland M, Enk L, Hammarberg K, et al. Use of a vaginal transducer for oocyte retrieval in an IVF/ET program. J Clin Ultrasound 1987; 15:245.
23. Wood C. Follicle aspiration. In: Gardner DK, Weissman A, Howles C, Shoham Z, eds. Textbook of Assisted Reproductive Techniques: Laboratory and Clinical Perspectives. London: Taylor & Francis, 2004:631.
24. Ditkoff EC, Plumb J, Selkic A, et al. Anesthesia practices in the United States common to in vitro fertilization (IVF) centers. J Assist Reprod Genet 1997; 14:145.
25. Bokhari A, Pollard BJ. Anaesthesia for assisted conception: a survey of UK practice. Eur J Anaesthesiol 1999; 16:225.
26. Corson SL, Batzer FR Gocial B, et al. Is paracervical block anesthesia for oocyte retrieval effective? Fertil Steril 1994; 62:133.
27. Ng EH, Tang OS, Chui DK, et al. A prospective, randomized, double-blind and placebo-controlled study to assess the efficacy of paracervical block in the pain relief during egg collection in IVF. Hum Reprod 1999; 14: 2783.
28. Ng EH, Chui DK, Tang OS, et al. Paracervical block with and without conscious sedation: a comparison of the pain levels during egg collection and the postoperative side effects. Fertil Steril 2001; 75:711.
29. Stener-Victorin E, Waldenström U, Nilsson L, et al. A prospective randomized study of electro-acupuncture versus alfentanil as anaesthesia during oocyte aspiration in in-vitro fertilization. Hum Reprod 1999; 14:2480..

30. Stener-Victorin E, Waldenström U, Andersson SA, et al. Reduction of blood flow impedance in the uterine arteries of infertile women with electro-acupuncture. Hum Reprod 1996; 11:1314.
31. Gejervall AL, Stener-Victorin E, Moller A, et al. Electro-acupuncture versus conventional analgesia: a comparison of pain levels during oocyte aspiration and patients' experiences of well-being after surgery. Hum Reprod 2005; 20:728.
32. Stener-Victorin E. The pain-relieving effect of electro-acupuncture and conventional medical analgesic methods during oocyte retrieval: a systematic review of randomized controlled trials. Hum Reprod 2005; 20:339.
33. Kingsland CR, Taylor CT, Aziz N, Bickerton N. Is follicular flushing necessary for oocyte retrieval? A randomized trial. Hum Reprod 1991; 6:382.
34. Tan SL, Watersone J, Wren M, et al. A prospective randomized study comparing aspiration only with aspiration and flushing for transvaginal ultrasound-directed oocyte recovery. Fertil Steril 1992; 58:356.
35. Bagtharia S, Haloob AR. Is there a benefit from routine follicular flushing for oocyte retrieval? J Obstet Gynaecol 2005; 25:374.
36. El-Shawarby S, Margara R, Trew G, A review of complications following transvaginal oocyte retrieval for in vitro fertilization. Hum Fertil (Camb) 2004; 7:127.
37. Tureck RW, Garcia CR, Blasco L, Perioperative complications arising after transvaginal oocyte retrieval. Obstet Gynecol 1993; 81:590.
38. Dicker D, Ashkenazi J, Feldberg D, Severe abdominal complications after transvaginal ultrasonographically guided retrieval of oocytes for in vitro fertilization and embryo transfer. Fertil Steril 1993; 59:1313.
39. Serour GI, Abulghar M, Monsorv R, Complications of medically assisted conception in 3,500 cycles. Fertil Steril 1998; 70:638.
40. Govaerts I, Deverker D, Delbavere A, Short-term medical complications of 1500 oocyte retrievals for in vitro fertilization and embryo transfer. Eur J Obstet Gynecol Reprod Biol 1998; 77:239.
41. Azem F, Wolfey Y, Botchan A, Massive retroperitoneal bleeding: a complication of transvaginal ultrasonography-guided oocyte retrieval for in vitro fertilization-embryo transfer. Fertil Steril 2000; 74:405.
42. Ashkenazi J, Farhi S, Dicker D, Acute pelvic inflammatory disease after oocyte retrieval: adverse effects on the results of implantation. Fertil Steril 1994; 61:526.
43. van Os HC, Roozenburg BJ, Janssen-Caspers HA, Vaginal disinfection with povidon iodine and the outcome of in-vitro fertilization. Hum Reprod 1992; 7:349.

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In Vitro Maturation of Oocytes

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INTRODUCTION

Since the first live birth resulting from in vitro fertilization (IVF) was reported 26 years ago (1), over two million live births have been reported as a result of IVF. IVF success rates have steadily improved over the years (2,3) and in many leading IVF centers today, the live-birth rate per cycle in women younger than 35 years may approach 50% (Table 1). Conventional IVF treatment requires that the ovaries be stimulated with gonadotropins, which contain follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in order to increase the number of mature oocytes retrieved, the number of embryos available for transfer, and, consequently, to improve pregnancy rates. Using controlled ovarian stimulation protocols, the success rates of IVF treatment have steadily increased and the results of many leading IVF centers today exceed those of spontaneous conceptions in healthy, fertile couples (3). However, ovarian stimulation protocols are associated with high costs, daily injections of gonadotropins and close monitoring, and carry a considerable risk of causing ovarian hyperstimulation syndrome (OHSS) (4). Although mild or moderate degrees of OHSS may not be very dangerous, severe OHSS may be associated with significant morbidity. Patients with polycystic ovaries (PCO) or polycystic ovarian

Table 1 Results of Fresh In Vitro Fertilization (IVF) Cycles Including IVF and IVF-Intracytoplasmic Sperm Injection Excluding Oocyte Donation Cycles

Age group	<35	35–37	38–40
Cycles started (% of total)	150 (33.6)	123 (27.6)	110 (24.7)
Cycles cancelled	6	6	2
Oocytes collected (mean)	14.4	14.0	12.0
Embryos transferred (mean)	2.6	2.9	3.3
Pregnancy rate per cycle started (%)	60.0	48.8	41.8
Pregnancy rate per embryo transfer (%)	65.7	53.1	45.1
Implantation rate per embryo (%)	36.6	24.5	15.1
Live birth rate per started cycle (%)	46.0	33.3	25.5
Live birth rate per embryo transfer (%)	50.4	36.3	27.5
Number of babies born	94	57	36
Singletons	46	25	22
Twins	21	16	7
Triplets	2	0	0

Source: McGill Reproductive Center.

syndrome (PCOS) are particularly prone to develop OHSS with an incidence of up to 6% (5). The most severe manifestation of OHSS involves massive ovarian enlargement and multiple cysts, hemoconcentration, and third-space accumulation of fluid. The syndrome may be complicated by renal failure and oliguria, hypovolemic shock, thromboembolic episodes, and adult respiratory distress syndrome which, in extreme cases, can even be fatal. Despite many years of clinical experience, no precise methods have been developed that will completely prevent severe OHSS after ovarian stimulation (6) and the only certain method is to avoid stimulating the ovaries with exogenous FSH. Some patients may also be deterred by the suggested association between multiple repeated cycles of ovarian stimulation and potential increased incidence of malignant diseases, a worrisome but unproven association (7). Avoiding ovarian stimulation and collection of immature oocytes would eliminate the risk of OHSS. Indeed, research on immature oocytes and their maturation was conducted as early as the mid-1930s (8).

OOCYTE MATURATION IN VIVO AND IN VITRO

Follicle Development and Oocyte Maturation In Vivo

The development of human oocytes is arrested at the prophase I stage of meiosis during fetal life. At birth, there are approximately one million primordial follicles in the ovaries (9), each of which consists of an oocyte surrounded by a few flattened pregranulosa cells enclosed by a basement

membrane. Although large numbers of follicles can leave the primordial pool and begin to grow, very few will be selected to mature and to ovulate for potential fertilization. Follicles respond to rising levels of gonadotropins by growing and fully maturing, then being released into the fallopian tube by ovulation only after the onset of puberty. During a woman's reproductive life, only about 400–500 mature oocytes will be released from the ovaries for potential fertilization. The process of follicular development within the ovary is directly influenced by gonadotropins, namely FSH and LH. From the growing cohort of antral follicles, only a portion is able to respond to the rising levels of FSH; consequently, a large number of follicles die at the early antral stage of development. During the early antral stage, the follicle has multilaminar granulosa cell layers and acquires vascularized, distinct layers of thecal cells that are separated from the granulosa cells by the basement membrane. It appears that approximately 20 antral follicles are selected and continued through the preovulatory stages of development during each menstrual cycle (10). During the later antral stage of follicular development, granulosa cells rapidly proliferate and differentiate into two populations, namely the mural granulosa cells that are adjacent to the basement membrane and the cumulus cells that surround the oocyte. Gonadotropins (FSH and LH) are necessary for follicular development in vivo, and both these hormones use the cyclic adenosine monophosphate pathway system as the intracellular second messenger. In addition, there are many other growth factors and cytokines that modulate the actions of gonadotropins; the follicle-enclosed oocyte being, as mentioned previously, arrested at the prophase stage of the first meiotic division. The resumption of the first meiotic division occurs in preovulatory follicles following the preovulatory LH surge. The nuclear membrane dissolves and the chromosomes progress from the metaphase I to the telophase I stage. The dissolution of the nuclear membrane is known as germinal vesicle breakdown. After the first meiotic division, which is characterized by the extrusion of the first polar body, the second meiotic division begins and a secondary metaphase plate (metaphase II) is formed. Therefore, oocyte maturation is defined morphologically as the reinitiation and completion of the first meiotic division from the germinal vesicle stage to the metaphase II stage with accompanying cytoplasmic maturation necessary for oocyte fertilization and early embryonic development. Oocytes that have not reached the metaphase II stage cannot be fertilized and undergo embryo cleavage. Knowledge of meiosis at the molecular level has accumulated rapidly in the last two decades. A major breakthrough was the discovery of a non-specific factor, the maturation-promoting factor, which is responsible for the G2 to the M-phase transition of the cell cycle (11). Molecular characterization of the maturation-promoting factor has shown that the active form is a protein dimmer composed of catalytic p34cdc serine/threonine kinase and regulatory cyclin B subunits.

In Vitro Maturation Oocytes

Although it is clear that the LH surge triggers the resumption of meiosis in vivo, cumulus–oocyte complexes can be spontaneously induced to resume meiosis when they are released from follicles into culture in vitro. Therefore, the action of endocrine factors affecting oocyte maturation in vitro may be quite different from in vivo conditions. Immature oocytes, with or without surrounding cumulus cells, can be matured to the metaphase II stage; however, the capacity of early embryonic development from the denuded oocytes is questionable. The beneficial effects of cumulus cells on early embryonic development have been reported in many species including humans (11). The actions of endocrine, paracrine, and autocrine factors that control oocyte maturation in vitro, either directly or indirectly, are mediated by the cumulus cells. Although FSH and LH play an important part in the development and maturation of preantral, antral, and preovulatory follicles in vivo, these gonadotropins may not play the same role in promoting oocyte maturation in vitro. Currently, most in vitro maturation (IVM) protocols supplement FSH or LH in a culture medium for oocyte maturation. However, the effects of FSH or LH on oocyte maturation and subsequent fertilization as well as early embryonic development are still controversial. The idea of supplementing these hormones in a culture medium is based on their physiological role in oocyte maturation in vivo.

The contradictory reports that FSH or LH are major hormones involved in IVM may be related to cross-contamination of FSH with LH or LH with FSH, as each preparation is derived from urinary extracts (12). Although it has been reported that using a combination of recombinant FSH with recombinant LH in IVM of immature oocytes resulted in significantly higher developmental competence, as evidenced by increased development to the blastocyst stage compared with recombinant FSH alone or no gonadotropins (13), conclusive results require further study. In addition, recently Hreinsson et al. (14) showed that use of recombinant human chorionic gonadotropin (hCG) or recombinant LH is equally effective in promoting oocyte maturation in vitro, although there was no proper control group in their study to substantiate this conclusion. It was initially considered that FSH and LH probably act to induce oocyte maturation in in vitro conditions through an indirect action mediated by cumulus cells, because it is believed that there are no FSH or LH receptors on the oocytes (15). However, recent reports (16,17) indicate that messenger RNA for FSH and LH receptors is present in mouse and human oocytes, zygotes, and preimplantation embryos, indicating a potential role for gonadotropins in the modulation of meiotic resumption and the completion of oocyte maturation. In addition, it has been known that culture medium supplemented with a physiological concentration of FSH or LH stimulates steroid secretions (estradiol and progesterone) from cultured granulosa and cumulus

cells (18). Therefore, it is likely that one of the actions of gonadotropins is mediated by either estradiol or progesterone, which may control oocyte maturation in vitro. A recent report indicated that LH-receptor formation in the cumulus cells surrounding porcine oocytes plays an important role in oocyte cytoplasmic maturation (19). However, its importance in oocyte maturation in vitro and how this action is linked to other signal transduction (pathways) are still largely unknown.

Estradiol and progesterone are mediators of normal mammalian ovarian function. Inhibition of steroid synthesis in whole cultured follicles impairs the subsequent fertilization and developmental capacity of oocytes in sheep (20). The presence of estradiol in the culture medium of in vitro matured human oocytes had no effect on the progression of meiosis but improved fertilization and cleavage rates (21). However, it may not be necessary to add estradiol to the oocyte maturation medium when the oocytes are cultured with cumulus cells because the culture medium supplemented with gonadotropins stimulates estradiol secretion from the granulosa and cumulus cells during culture in vitro (18). Little information is currently available about how progesterone contained in the culture medium affects oocyte maturation. However, we have found that progesterone has a negative effect on bovine oocyte maturation in vitro, and it is well known that many growth factors are contained in follicular fluid. These growth factors must be secreted from the granulosa and cumulus cells that respond to gonadotropins and subsequently act on the oocyte via paracrine and autocrine pathways. Although a growing number of studies have indicated that growth factors produce beneficial effects on oocyte maturation, it seems that only denuded oocytes require the supplementation of growth factors in the culture medium for proper oocyte maturation (22). This suggests that the granulosa and cumulus cells can secrete some growth factors during culture and play some functional roles during oocyte maturation in vitro. In practice, the culture medium is also supplemented with the patient's own serum or human serum albumin as a protein source. Both serum and human serum albumin are a rich source of growth factors. Therefore, it is not necessary to add growth factors to the IVM medium for oocyte maturation in vitro, especially when the IVM medium contains serum or human serum albumin.

IN VITRO MATURATION OF OOCYTES IN INFERTILITY TREATMENTS

The research into maturation of immature oocytes initiated by Pincus and Enzmann (8) and continued by Edwards et al. (23) was not incorporated as a treatment for human infertility until 1991. Cha et al. (24) reported that human follicular oocytes were harvested from unstimulated ovaries during gynecological surgery, matured in vitro, then fertilized, and five embryos

were transferred to a woman with premature ovarian failure. The recipient subsequently delivered healthy triplet girls. Trounson et al. (25) further suggested that immature oocyte recovery could be developed as a new method for the treatment of women with infertility due to PCO because the oocytes of these patients retain their maturational and developmental competence. However, the initial reported IVM pregnancy rates were low. Our group demonstrated that priming with hCG 36 hours prior to immature oocyte collection significantly improved the maturation rate, and the pregnancy rate exceeded 30% (26,27). IVM was initially considered as a treatment for patients with PCOS, but the indications are now expanding to include various other fertility problems.

IVM of Oocytes from Women with PCOS

PCOS is a very heterogeneous syndrome, often first diagnosed when the patient presents complaining of infertility; approximately 75% of these women suffer infertility due to anovulation. The majority of women with anovulation or oligo ovulation due to PCOS have menstrual irregularities, usually oligo- or amenorrhea, associated with clinical and/or biochemical evidence of hyperandrogenism. In almost all these patients, ultrasonic scan of the ovaries typically reveals numerous antral follicles (28,29). Fertility treatments for women with PCOS include lifestyle management, administration of insulin-sensitizing agents, laparoscopic ovarian drilling, ovulation induction, ovarian stimulation, and IVF. As previously mentioned, this group of patients has an increased risk of severe OHSS from gonadotropin stimulation compared with women who have normal ovaries (5,30). The risk of multiple-follicle ovulation and subsequent multiple pregnancies is also of crucial importance (5,31). However, the high number of antral follicles in patients with PCO makes them prime candidates for IVM treatment, even if the appearance of PCO in the scan is not associated with an ovulation disorder. Indeed, the main determinant clinically of success rates of IVM treatment is antral follicle count. When hCG priming is used before oocyte retrieval, it has been found that immature oocytes retrieved from normal ovaries, PCO, or women with PCOS have a similarly high maturation, fertilization, and cleavage potential (32). However, although the implantation rate was lower, the live-birth rates were not significantly different and, as expected, the OHSS rate was significantly lower in the IVM group. These results suggested that IVM is a promising alternative to conventional IVF treatment for women with PCO or a high antral follicle count who require assisted conception (33).

IVM for High Responders to Gonadotropin Stimulation

When patients receiving gonadotropins hyper-respond to treatment, there are no precise methods to completely prevent severe OHSS. However, the

risk can be reduced by withholding the ovulation-inducing trigger of hCG (34). Thus, in conventional ovarian stimulation for IVF where there has been an over-response and there is a high chance of developing OHSS, the cycle would be cancelled. Immature oocyte retrieval followed by IVM and IVF may provide an alternative to cancellation of these cycles. Initially, one live birth was reported from immature oocytes collected from a patient at substantial risk of developing OHSS (35). More recently, Lim et al. (36) reported 17 patients with a high risk of developing OHSS during the course of their IVF cycles. Instead of canceling the cycles, they undertook immature oocyte collection followed by IVM. hCG was administered 36 hours before oocyte collection when the leading follicle had reached a mean diameter of 12–14 mm and indeed 11.6% of the oocytes had already reached the metaphase II stage at collection. Eight out of 17 (47.1%) clinical pregnancies were achieved in this group of patients. Even though the safest method of preventing OHSS is to withhold hCG administration (34), no cases of OHSS were reported among these patients, who were at a high risk of developing the syndrome (36). To date, more than 30 healthy live births have been reported from this group of patients following oocyte retrieval and IVM treatment (personal communication). Therefore, patients who are at risk of developing OHSS during controlled ovarian hyperstimulation can resort to immature oocyte retrieval followed by IVM as an alternative to canceling the cycle.

IVM for Poor Responders

Poor response to gonadotropin stimulation occurs more often in older women but may also be present in young women, including those with normal endocrine profiles as well as those with abnormal endocrine parameters—namely, high baseline FSH and estradiol (E_2) levels—known to be associated with poor response. Some poor responders appear to respond to stimulation but have a low estrogen level, whereas others have few or slow-growing follicles. Normally, these patients require prolonged stimulation and higher doses of gonadotropins. They also experience a high cancellation rate because of the smaller number or size of follicles. Many different ovarian stimulation protocols have been tried for treatment of poor responders in IVF. No single protocol seems to benefit all poor responders and treatment continues to challenge those involved in IVF programs (37–39). Although oocyte donation would be the ideal treatment for these patients, some may refuse this option because they would prefer to try using their own oocytes. In these cases, poor responders to previous gonadotropin stimulation may benefit from immature oocyte collection from unstimulated ovaries. In a study by Child et al. (40), eight women with a previous poor response to IVF underwent oocyte collection without ovarian stimulation. hCG was administrated 36 hr before collection. An average of 2.3 immature

oocytes were collected and an average of 1.7 matured in vitro. Six of the eight women underwent embryo transfer of 1–3 embryos (average of 1.7); one patient became pregnant and subsequently delivered. The number of embryos produced and available for embryo transfer was similar to that for previous IVF treatments (40). During ovarian stimulation, the small number and size of follicles often warrant cancellation of the cycle. As an alternative to cancellation, immature oocytes could be collected from the stimulated but unresponsive ovaries and then matured in vitro. Such pregnancies were first reported after cryopreservation of in vitro matured oocytes (41). Liu et al. (42) reported eight cases of immature oocyte collection in young patients who had shown poor response to gonadotropin stimulation; three pregnancies were achieved. In another report (43), 41 patients were identified as being resistant to gonadotropin stimulation as the follicles did not grow despite increasing the dosage of gonadotropins. To optimize the successful pregnancy rate among these poor responders, hCG was administered and oocyte retrieval performed 36 hours later because at least some in vivo matured oocytes could be collected after hCG administration. This indicates that immature oocyte retrieval followed by IVM is a possible alternative to cancellation of the treatment cycle in women with poor response following ovarian stimulation (41–43). Based on the results of these preliminary studies, it seems that IVM is a possible option for patients with a poor ovarian response in an ongoing stimulated IVF cycle or with a history of a previous low response to gonadotropin stimulation. Although IVM does not always produce better results than conventional IVF in these cases, it will at least give comparable results without the need for prolonged stimulation with large doses of gonadotropins.

Oocyte Donation

Oocyte donation has become a standard treatment for women with diminished ovarian reserve and/or who are of advanced reproductive ages women affected by, or who are carriers of a significant genetic defect; and women with poor oocyte and/or embryo quality (44). Oocyte donation results in a high pregnancy rate for patients with an otherwise grave reproductive prognosis; the accumulated pregnancy rate may increase up to 94.8% after four transfers (45). The risk of OHSS, complications associated with oocyte collection, and concern about the inconvenience of a large number of hormone injections as well as possible long-term side effects (46,47) may deter some potential oocyte donors. Indeed, results of a recent survey indicate that three-quarters of potential donors changed their mind about donating after receiving information on the procedures involved (48). Avoiding ovarian stimulation would obviously eliminate the associated risks to oocyte donors and would drastically reduce the costs of donation cycles (49). As discussed earlier, the first reported IVM pregnancy was conceived

from immature oocytes retrieved and donated to a woman with premature ovarian failure (24). At our center, 12 oocyte donors (age 29 ± 4) with high antral follicle counts (29.6 ± 8.7) underwent immature oocyte collection without ovarian stimulation. A mean of 12.8 ± 5.1 germinal vesicle (GV) oocytes were collected, 68% matured and underwent intracytoplasmic sperm injection (ICSI). A total of 47 embryos were transferred to 12 recipients and six (50%) conceived, of which four have resulted in live births (Holzer H, Chian RC, Scharf E, Tan SL. IVM oocyte donors: oocyte donation without ovarian stimulation, in preparation). Therefore, collecting immature oocytes from a donor's unstimulated ovaries in oocyte donation programs seems prudent and worthwhile.

IVM and Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) is a procedure whereby embryos produced by couples who are at risk of having children with an inherited disease or genetic defect, or by patients who have had three or more unexplained miscarriages, can be tested prior to implantation. Couples can therefore choose to have only those embryos diagnosed as being unaffected implanted in the woman's uterus, thus improving the chances of a successful pregnancy. IVF is normally necessary for patients who elect to undergo this procedure in order to generate multiple embryos for genetic analysis. We have recently used IVM as an alternative for selected patients with PCO/PCOS who require PGD so as to avoid the side effects of fertility-drug administration and avoid the risk of OHSS. We recently treated a 35-year-old patient with recurrent miscarriage who had been unsuccessfully treated with two IUI and two IVF cycles in Germany. We collected one MII and 14 GV oocytes and biopsied eight embryos generated. After the transfer of two normal embryos following aneuploidy screening, she became pregnant and we had the world's first live birth after combined IVM and PGD (50).

Fertility Preservation

In the modern era, cancer is a common lethal disease. It was estimated that in 2003 over 650,000 new cases of female cancer were diagnosed in the United States. An encouraging fact is that during the last three decades, a tremendous improvement in the success rates of cancer treatments has resulted in a steady increase in the survival rates. Although the agents used for treatment of many types of cancer are successful in up to 95% of patients, they unfortunately carry a considerable risk of causing the loss of future fertility potential. Because many cancer patients are in the early reproductive age group, they would like to have the option to preserve their fertility potential to allow them to lead a future normal, healthy life. Mature

oocytes could be harvested from ovaries of cancer patients after controlled ovarian hyperstimulation. However, there are two major drawbacks associated with conventional IVF; first, the time interval needed for IVF ranges from 2 to 6 weeks beginning with the patient's next menstrual period, which may sometimes be too long due to the natural course of the malignant disease without therapy. Second, ovarian hyperstimulation is associated with high estradiol levels which may not be safe in some cases of estrogen-sensitive breast cancer. Ovarian stimulation for oocyte collection could be totally avoided by collecting immature oocytes (51). We recently reported the retrieval of immature oocytes from unstimulated ovaries before gonadotoxic therapy for oocyte vitrification purposes (52). This resulted in the successful preservation of fertility with no delay in chemotherapy, no surgery, and no necessity for hormonal stimulation. Since that report, 26 cancer patients have undergone immature and mature oocyte collection from totally unstimulated ovaries. Collection can be performed during the follicular phase prior to ovulation for normal ovulating patients and on almost any given day for PCOS patients (51,52). The immature oocytes are then matured in vitro. The oocytes can either be fertilized utilizing the partner's sperm and the resulting embryos cryopreserved or, if the patient does not have a partner, the mature oocytes are vitrified. Vitrification of oocytes collected from unstimulated ovaries seems like a promising procedure for preservation of fertility, as this technique avoids hormonal stimulation and is not associated with considerable delay in cancer treatment. Vitrification of the matured oocytes will hopefully yield much higher oocyte survival and pregnancy rates than do the currently used methods (51).

OUTLINE OF AN IVM TREATMENT CYCLE

Ultrasound

A baseline scan is performed between days 2 and 5 of the menstrual cycle. If the patient is amenorrheic, a withdrawal bleed with progestogens is induced. At the baseline scan, ovarian volume, ovarian stromal blood flow velocity, number of antral follicles, size of the follicles, endometrial thickness, and any ovarian or uterine abnormality are recorded. The antral follicle count, ovarian volume, and ovarian stromal maximal blood velocity are all predictors of the number of oocytes retrievable; however, we have found that when the other factors were controlled by multiple regression analysis, the antral follicle count was the only significant predictor (53). A second scan is performed on days 6–8 of the cycle to repeat all the above-described measurements. Along with others we had recently reported that when a dominant follicle is present, atresia does not occur in the other nondominant follicles (54–56). Therefore, we no longer cancel the procedure in patients with a dominant follicle.

Priming with hCG and Pretreatment with FSH

Some studies suggest that pretreatment with FSH during the early follicular phase will enhance the number of oocytes retrieved and their rate of maturation (57), whereas others have shown no benefit from pretreatment with FSH (58,59). At our center, we do not stimulate the ovaries with FSH prior to an IVM oocyte collection.

We have shown that hCG priming prior to IVM oocyte collection increases the maturation rate of oocytes in vitro (27) and we administer 10,000 IU of hCG 36 hours prior to collection. A prospective, randomized controlled trial demonstrated no improvement in oocyte maturation rates with 20,000 IU of hCG compared with 10,000 IU of hCG; therefore, there is no benefit from the higher dose (60).

Immature Oocyte Retrieval

Oocyte retrieval is done under spinal anesthesia or intravenous sedation using fentanyl and midazolam (1–2 mg). Intravenous fentanyl is administered at intervals of 15–20 min up to a total dose of 150–200 mg. Local infiltration of bupivacaine 0.5% in the vagina reduces the discomfort of multiple needle punctures. Retrieval is performed under ultrasound guidance with a 19-G, single-lumen aspiration needle. The aspiration pressure is reduced to 7.5 kPa. The follicular fluid is collected in culture tubes containing 0.9% saline with 2 U/mL of heparin. Because immature oocytes are enclosed in tightly packed cumulus cells, curettage of the follicle wall will dislodge the cumulus oocyte complex. In an immature oocyte collection, multiple needle punctures are needed. Because the aspiration pressure is low and a small-gauged needle is used, the bloodstained aspirate may often block the needle. Therefore, the needle is withdrawn from the vagina after aspirating a few follicles to flush and clear any blockage. The procedure is repeated until all follicles seen are aspirated.

Maturation In Vitro and Fertilization

Immature oocytes are incubated in a culture dish containing maturation medium. The maturation medium is supplemented with 75 mIU/mL of FSH and LH. The oocytes are cultured at 37°C in an atmosphere of 5% carbon dioxide and 95% air with high humidity. Oocytes are checked for maturity 24 and 48 hours after culture. The oocytes are denuded of granulosa cells, and mature oocytes (detected by the presence of an extruded polar body) are fertilized by ICSI. ICSI is performed for in vitro matured oocytes because it reduces the risk of unexpected poor fertilization as compared with IVF. However, it has been demonstrated that ICSI may not always be essential for the fertilization of in vitro matured human oocytes collected from unstimulated ovaries when the sperm parameters are normal (61). After

ICSI, the oocytes are transferred into 1 mL of IVF medium in a tissue culture dish. Fertilization is assessed 18 hours after ICSI by examining the oocytes for the appearance of two distinct pronuclei and two polar bodies.

Embryo Transfer

The fertilized oocytes are further cultured up to day 2 or 3, and then embryo transfer is performed. Assisted hatching is performed to avoid reduced implantation due to a hardened zona pellucida. When a large number of embryos have been formed, alternative approaches could be either an extended culture to the blastocyst stage or a double transfer (62). A double transfer is performed on day 2 or 3 and a blastocyst transfer on day 5 or 6. The embryo transfer technique is the same as that employed for conventional IVF.

Endometrial Preparation and Luteal Support

To achieve optimum endometrial growth, exogenous estradiol 17β (micronized) is started on the day of retrieval. The dosage is determined by endometrial thickness measured on the day of retrieval. If the endometrial thickness is less than 6 mm, then 12 mg a day is started; if the thickness is between 6 and 8 mm, then 10 mg a day is started and if the thickness is more than 8 mm, then 6 mg is used, all in three divided doses. Recently, we have begun administering the estradiol treatment even before oocyte collection, when an extremely thin endometrium (i.e., <4 mm) is recorded on an ultrasound scan prior to the collection. We are currently investigating an alternative approach whereby the in vitro matured oocyte is vitrified when the endometrial lining is thin. The endometrium is then prepared in an artificial cycle and, once it reaches 8 mm, the oocytes are thawed, fertilized, and transferred. In an IVM treatment cycle, luteal support is started on the day that oocyte maturation is achieved and ICSI is performed, with daily intramuscular injections of progesterone in oil or Prometrium (Schering Canada) 200, tid. Estradiol and progesterone supplementation is continued until the 12th week of pregnancy (Fig. 1).

IVM TREATMENT OUTCOME

Pregnancy rates with IVM are correlated with the number of immature oocytes retrieved. In women younger than 35 years from whom we retrieved more than 10 immature GV oocytes, we have achieved a clinical pregnancy rate of 38% per cycle. With an oocyte retrieval rate of more than 50% from the follicles present, women with 20 or more follicles at the baseline scan for IVM would be the best candidates for IVM. Our implantation rates are approximately 12%. As with IVF, clinical pregnancy and implantation rates decrease with increasing age. In women younger than 35 years, we have

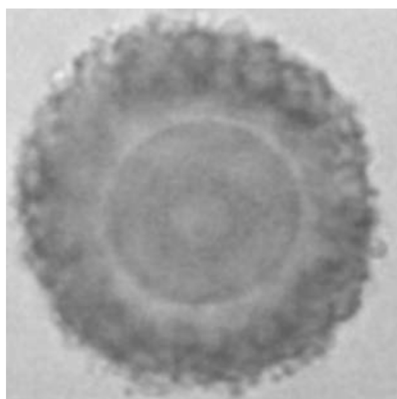

Figure 1 Immature human oocyte retrieved from a follicle at 4 mm in diameter. The oocyte with several layers of compacting cumulus cells. *Source*: From R.C. Chian, with permission.

achieved a clinical pregnancy rate of 38% per oocyte retrieval and an implantation rate of 13%. In women between 36 and 40 years old, the clinical pregnancy rate is 21% per retrieval and the implantation rate 5% (Table 2). Based on more than 1000 IVM cycles in four centers performing IVM cycles with hCG priming before oocyte collection, the pregnancy rates reached 30–35% and the implantation rates 10–15% (63). Some authors have expressed concerns regarding the safety of IVM, especially in relation to imprinting gene disorders (64–66). In various published series, no increased

Table 2 Results of Fresh In Vitro Maturation Cycles Excluding Oocyte Donation Cycles

Age	<35	35–40
Oocyte collections	46	19
Embryo transfers	45	17
Number of oocytes collected (mean)	15.0	13.2
Number of embryos transferred (mean)	3.7	4.5
Pregnancy rate per collection (%)	41.3	21.1
Pregnancy rate per embryo transfer (%)	42.2	23.5
Implantation rate (%)	14.4	5.2
Live birth rate per collection (%)	21.7	15.8
Live birth rate per embryo transfer (%)	22.2	17.6
Number of babies born	12	3
Singletons	6	3
Twins	3	0

Source: McGill Reproductive Center.

rates of congenital malformations have been reported with IVM (67,68). A recent analysis of the obstetrical, neonatal, and infant outcome in our IVM conceptions showed pregnancy rates of 73% singleton, 24% twin, and 2.7% triplet. The median gestation age was 39 weeks for singletons and 37 weeks for multiple pregnancies. There were only two malformations, including a ventriculo septal defect and a congenital dislocation of the hips (69). There was no increased relative risk of malformations when IVM pregnancies were compared with IVF and spontaneous pregnancies (70). Similar reassuring results have been published by others (71).

CONCLUSIONS AND FUTURE CONSIDERATIONS

IVM is an evolving technique which was initially indicated for patients with PCO because it avoids the necessity of ovarian stimulation, thus eliminating the risk of OHSS; however, this technology should now be extended to other causes of infertility. IVM cycles require less monitoring and fewer clinic visits, lessening the burden on patients and further reducing costs which are already lower due to the lack of gonadotropin stimulation. IVM treatment could be offered as an alternative to IVF to all infertile patients who have a high number of antral follicles, and who are at increased risk of developing OHSS. For patients who are over-responding to ovarian stimulation, treatment can be converted to IVM when the follicles are still small. Poor responders may benefit from IVM treatment if they refuse oocyte donation, as they do not need to receive large doses of gonadotropins. For cancer patients seeking to preserve their fertility, IVM enables them to avoid hormonal stimulation and saves precious time. IVM combined with oocyte vitrification is also an option for fertility preservation for women who wish to delay childbearing, and IVM may also be a boon to oocyte donation programs by eliminating the need for ovarian stimulation of potential donors. The pregnancy rates are encouraging and, at 30–35% per cycle, are comparable to the IVF results of many IVF centers. However promising the results may be, the implantation rates need to be improved and much further progress is needed. With the development of fluorescent in situ hybridization and spectral karyotyping for genetic analysis of oocyte and polar body chromosomes, it may soon be possible to assess the frequency of IVM oocyte meiotic errors. These and other predictors of embryo competence, if developed, will lead to improved implantation rates. Culture conditions will probably also be improved in future thanks to the vast amount of research currently being undertaken and the progressing clinical experience. The improved implantation rates will enable us to transfer fewer embryos and reduce the multiple gestation rate. Long-term follow-up of the children conceived through IVM is of crucial importance in establishing this treatment modality. Altogether, IVM is a promising technique which will have increasing applications in the coming years.

REFERENCES

1. Steptoe PC, Edwards RG. Birth after re-implantation of a human embryo. Lancet 1978; 2:336.
2. Tan SL, Royston P, Campbell S, et al. Cumulative conception and live birth rates after in vitro fertilization. Lancet 1992; 339:1390–1394.
3. Engmann L, Maconochie N, Bekir JS, Jacobs HS, Tan SL. Cumulative probability of clinical pregnancy and live birth after a multiple cycle IVF package: a more realistic assessment of overall and age-specific success rates? Br J Obstet Gynecol 1999; 106:165–170.
4. Brinsden P, Wada I, Tan SL, Balen A, Jacobs HS. Diagnosis, prevention and management of ovarian hyperstimulation syndrome. Br J Obstet Gynecol 1995; 102:767–772.
5. MacDougall MJ, Tan SL, Balen A, Jacobs HS. A controlled study comparing patients with and without polycystic ovaries undergoing in vitro fertilization. Hum Reprod 1993; 8:233–237.
6. Tan SL, Balen A, Hussein EL, Campbell S, Jacobs HS. The administration of glucocorticoids for the prevention of ovarian hyperstimulation syndrome in IVF: a prospective randomized study. Fertil Steril 1992; 58:378–383.
7. Brinton LA, Moghissi KS, Scoccia B, Westhoff CL, Lamb EJ. Ovulation induction and cancer risk. Fertil Steril 2005; 83:261–274.
8. Pincus G, Enzmann EV. The comparative behavior of mammalian eggs in vivo and in vitro. I. The activation of ovarian eggs. J Exp Med 1935; 62:655–675.
9. Baker TG. Oogenesis and ovulation. In: Austin CR, Short RV, eds. Reproduction in Mammals 2nd ed. Cambridge: Cambridge University Press, 1982:17–45.
10. Hillier SG. Current concepts of the role of FSH and LH in folliculogenesis. Hum Reprod 1994; 9:188–191.
11. Cha KY, Chian RC. Maturation in vitro of immature human oocytes for clinical use. Hum Reprod Update 1998; 4:103–120.
12. Bevers MM, Fieleman SJ, Van den Hurk R, Izadyar F. Regulation and modulation of oocyte maturation in the bovine. Theriogenology 1997; 47:13–22.
13. Anderiesz C, Ferraretti A, Magli C, et al. Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development in vitro. Hum Reprod 2000; 15:1140–1148.
14. Hreinsson J, Rosenlund B, Friden B, et al. Recombinant LH is equally effective as recombinant hCG in promoting oocyte maturation in a clinical in vitro maturation programme: a randomized study. Hum Reprod 2003; 18:2131–2136.
15. Dekel N, Lawrence TS, Gilula NB, Beers WH. Modulation of cell-to-cell communication in the cumulus–oocyte complex and regulation of oocyte maturation by LH. Dev Biol 1981; 86:356–362.
16. Patsoula E, Loutradis D, Drakakis P, et al. Expression of mRNA for the LH and FSH receptors in mouse oocytes and preimplantation embryos. Reproduction 2001; 121:455–461.
17. Patsoula E, Loutradis D, Drakakis P, et al. Messenger RNA expression for the follicle-stimulating hormone receptor and luteinizing hormone receptor in human oocytes and reimplantation-stage embryos. Fertil Steril 2003; 79:1 187–1193.

18. Chian RC, Ao A, Clarke HJ, Tulandi T, Tan SL. Production of steroids from human cumulus cells treated with different concentrations of gonadotropins during culture in vitro. Fertil Steril 1999; 71:61–66.
19. Shimada M, Nishibori M, Isobe N, et al. Luteinizing hormone receptor formation in cumulus cells surrounding porcine oocytes and its role during meiotic maturation of porcine oocytes. Biol Reprod 2003; 68:1142–1149.
20. Moor RM, Polge C, Willadsen SM. Effects of follicular steroids on the maturation and fertilization of mammalian oocytes. J Embryol Exp Morph 1980; 56:319–335.
21. Tesarik J, Mendoza C. Nongenomic effects of 17β-estradiol on maturation of human oocytes: relationship to oocyte developmental potential. J Clin Endocrinol Metab 1995; 80:1438–1443.
22. Chian RC, Tan SL. Maturational and developmental competence of cumulus free immature human oocytes derived from stimulated and intracytoplasmic sperm injection cycles. Reprod Biomed Online 2002; 5:125–132.
23. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. Nature 1969; 221:632–635.
24. Cha KY, Koo JJ, Ko JJ, et al. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. Fertil Steril 1991; 55:109–113.
25. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil Steril 1994; 62:353–362.
26. Chian RC, Gulekli B, Buckett WM, Tan SL. Priming with human chorionic gonadotropin before retrieval of immature oocytes in women with infertility due to the polycystic ovary syndrome. N Engl J Med 1999; 341:1624–1626.
27. Chian RC, Buckett WM, Tulandi T, Tan SL. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. Hum Reprod 2000; 15:165–170.
28. Tan SL, Farhi J, Homburg R, Jacobs HS. Induction of ovulation in clomiphene resistant polycystic ovary syndrome with pulsatile GnRH. Obstet Gynecol 1996; 88:221–226.
29. Balen AH, MacDougall J, Tan SL. The influence of the number of embryos transferred in 1060 in vitro fertilization pregnancies on miscarriage rates and pregnancy outcome. Hum Reprod 1993; 8:1324–1328.
30. MacDougall J, Tan SL, Jacobs HS. In vitro fertilisation and ovarian hyperstimulation syndrome. Hum Reprod 1992; 7:597–600.
31. Homburg R. Management of infertility and prevention of ovarian hyperstimulation in women with polycystic ovary syndrome. Best Pract Res Clin Obstet Gynaecol 2004; 18:773–788.
32. Child TJ, Abdul-Jalil AK, Gulekli B, Tan SL. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. Fertil Steril 2001; 76:936–942.
33. Child TJ, Phillips SJ, Abdul-Jalil AK, Tan SL. A comparison of in vitro maturation and in vitro fertilization for women with polycystic ovaries. Obstet Gynecol 2002; 100:665–670.

34. Orvieto R. Can we eliminate severe ovarian hyperstimulation syndrome? Hum Reprod 2005; 20:320–322.
35. Jaroudi KA, Hollanders JMG, Sieck UV, Roca GL, El-Nour AM, Coskun S. Pregnancy after transfer of embryos which were generated from in vitro matured oocytes. Hum Reprod 1997; 12:857–859.
36. Lim KS, Son WY, Yoon SH, Lim JH. IVM/F-ET in stimulated cycles for the prevention of OHSS (Abstract). Fertil Steril 2002; 78:S10.
37. Nikolettos N, Al-Hasani S, Felberbaum R, et al. Gonadotropin-releasing hormone antagonist protocol: a novel method of ovarian stimulation in poor responders. Eur J Obstet Gynecol Reprod Biol 2001; 97:202–207.
38. Lashen H, Ledger W, Lopez-Baernal A, Barlow D. Poor responders to ovulation induction: is proceeding to in vitro fertilization worthwhile? Hum Reprod 1999; 14:964–969.
39. Surrey ES, Schoolcraft WB. Evaluating strategies for improving ovarian response of the poor responder undergoing assisted reproductive techniques. Fertil Steril 2000; 73:667–676.
40. Child TJ, Gulekli B, Chian RC, Abdul-Jalil K, Tan SL. In vitro maturation (IVM) of oocytes from unstimulated normal ovaries of women with a previous poor response to IVF. Fertil Steril 2000; 74:s45.34.
41. Check ML, Brittingham D, Check JH, Choe JK. Pregnancy following transfer of cryopreserved-thawed embryos that had been a result of fertilization of all in vitro matured metaphase or germinal stage oocytes, case report. Clin Exp Obstet Gynecol 2001; 28:69–70.
42. Liu J, Lu G, Qian Y, Mao Y, Ding W. Pregnancies and births achieved from in vitro matured oocytes retrieved from poor responders undergoing stimulation in in vitro fertilization cycles. Fertil Steril 2003; 80:447–449.
43. Chian RC, Son YW, Yoon SH, Lim JH, Tan SL. Pregnancies achieved from in vitro matured oocytes retrieved from poor responders in stimulated IVF cycles (Abstract). Hum Reprod 2004; 19:100i.
44. The American Society for Reproductive Medicine. Guidelines for oocyte donation. Fertil Steril 2004; 82:s13–s15.
45. Remohi J, Gartner B, Gallardo E, Yalil S, Simon C, Pellicer A. Pregnancy and birth rates after oocyte donation. Fertil Steril 1997; 67:717–723.
46. The American Society for Reproductive Medicine. Repetitive oocyte donation. Fertil Steril 2004; 82:s158–s159.
47. Bennett SJ, Waterstone JJ, Cheng WC, Parsons J. Complications of transvaginal ultrasound-directed follicle aspiration: a review of 2670 consecutive procedures. J Assist Reprod Genet 1993; 10:72–77.
48. Murray C, Golombok S. Oocyte and semen donation: a survey of UK licensed centres. Hum Reprod 2000; 15:2133–2139.
49. Scharf E, Chian RC, Abdul Jalil K, Sylvestre C, Buckett W, Tan SL, In vitro maturation of oocytes: a new option for donor oocyte treatment. Annual Meeting of the ASRM [abstr]. Fertil Steril 2004; 82(suppl 2):S14.
50. Ao A, Jin S, Rao D, Son WY, Chian RC, Tan SL. First successful pregnancy outcome after preimplantation genetic diagnosis for aneuploidy screening in embryos generated from natural-cycle in vitro fertilization combined with an in vitro maturation procedure. Fertil and Steril 2006; 85:1510.e9–1510.e11.

51. Holzer H, Tan SL. Fertility preservation in oncology. Minerva Gynecol 2005; 57:99–109.
52. Rao GD, Chian RC, Son WS, Gilbert L, Tan SL. Fertility preservation in patients undergoing cancer treatment. Lancet 2004; 363:1829.
53. Tan SL, Child TJ, Gulekli B. In vitro maturation and fertilization of oocytes from unstimulated ovaries: predicting the number of immature oocytes retrieved by early follicular phase ultrasonography. Am J Obstet Gynecol 2002; 186: 684–689.
54. Paulson RJ, Sauer MV, Francis MM, et al. Factors affecting pregnancy success of human in vitro fertilization in unstimulated cycles. Hum Reprod 1994; 9: 1571–1575.
55. Thornton MH, Francis MM, Paulson RJ. Immature oocyte retrieval: lessons from unstimulated IVF cycles. Fertil Steril 1998; 70:647–650.
56. Chian RC, Buckett WM, Abdul Jalil AK et al. Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential approach in infertility treatment. Fertil Steril 2004; 82:1675–1678.
57. Wynn P, Picton HM, Krapez JA, Rutherford JA, Balen AH, Gosden RG. Pretreatment with follicle stimulating hormone promotes the number of human oocytes reaching metaphase II by in vitro maturation. Hum Reprod 1998; 13:3132–3138.
58. Mikkelsen AL, Smith SD, Lindenberg S. In vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. Hum Reprod 1999; 14:1847–1851.
59. Trounson A, Anderiesz C, Jones G. Maturation of human oocytes in vitro and their developmental competence. Reproduction 2001; 121:51–75.
60. Gulekli B, Buckett WM, Chian RC, Child TJ, Abdul-Jalil AK, Tan SL. Randomized, controlled trial of priming with 10,000 IU versus 20,000 IU of human chorionic gonadotropin in women with polycystic ovary syndrome who are undergoing in vitro maturation. Fertil Steril 2004; 82:1458–1459.
61. Soderstron AV, Makinen S, Tuuri T, Suikkari AM. Favourable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. Hum Reprod 2005; 20:1534–1540.
62. Philips SJ, Dean NL, Buckett WM, Tan SL. Consecutive transfer of day 3 embryos and of day 5–6 blastocysts increases overall pregnancy rates associated with blastocyst culture. J Assist Reprod Genet 2003; 20:461–464.
63. Chian RC. In vitro maturation of immature oocytes for infertile women with PCOS. Reprod Biomed Online 2004; 8:547–552.
64. Albertini DF, Sanfins A, Combelles C. Origins and manifestations of oocyte maturation competencies. Reprod Biomed Online 2003; 6:410–415.
65. Fauser BCJ, Bouchard P, Coelingh JT, et al. Alternative approaches of IVF. Hum Reprod Update 2002; 8:1–9.
66. Hardy K, Wright CS, Franks S, Winston RM. In vitro maturation of oocytes. Br Med Bull 2000; 56:588–602.
67. Mikkelsen AL, Andersson A-M, Skakkebaek NE, Lindenberg S. Basal concentrations of estradiol may predict the outcome of in vitro maturation in regularly menstruating women. Hum Reprod 2001; 16:862–876.
68. Cha KY, Han SY, Chung HM, et al. Pregnancies and deliveries after in vitro maturation culture followed by in vitro fertilization and embryo transfer without

stimulation in women with polycystic ovary syndrome. Fertil Steril 2000; 73: 978–983.
69. Buckett WM, Chian R, Holzer H, Usher R, Tan SL. Congenital abnormalities and perinatal outcome in pregnancies following IVM, IVF, and ICSI delivered in a single center. In: Proceedings of the 61st Annual Meeting of the American Society for Reproductive Medicine, Montreal, Canada (Abstract 0-196). Fertil Steril 2005; 84(suppl 1):S80–S81.
70. Buckett WM, Chian RC, Barrington K, Dean N, Abdul-Jalil K, Tan SL. Obstetric, neonatal and infant outcome in babies conceived by in vitro maturation (IVM): initial five-year results 1998–2003 (Abstract O-331). In: Proceedings of the 60th Annual Meeting of the American Society for Reproductive Medicine, Philadelphia, Pennsylvania, U.S.A., October 16–20. Fertil Steril 2004; 82(suppl 2): S133.
71. Cha KY, Chung HM, Lee DR, et al. Obstetric outcome of patients with polycystic ovary syndrome treated by in vitro maturation and in vitro fertilization–embryo transfer. Fertil Steril 2005; 83:1461–1465.

9

Sperm Preparation for IVF and ICSI

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INTRODUCTION

Human spermatozoa at ejaculation are incapable of in vivo fertilization and must undergo maturational change during which they acquire the ability to fertilize oocytes. This process, known as capacitation, was described more than 50 year ago by both Austin (1) and Chang (2). Capacitation is prevented in ejaculated spermatozoa by at least one factor in seminal plasma (3). Additionally, prolonged exposure to seminal plasma can inhibit the ability of spermatozoa to undergo the acrosome reaction in vitro (4) and diminish their capacity to fertilize (5). In the female genital tract, motile spermatozoa separate themselves from seminal plasma, immotile spermatozoa, and debris by actively migrating through the cervical mucus. This active migration selects progressively motile spermatozoa and allows them to undergo capacitation. Due to the inhibitory effects of seminal plasma on sperm function, it is critical that spermatozoa used for clinical procedures such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) be separated from the seminal plasma as quickly as possible after ejaculation and liquefaction.

Although IVF started as a treatment for tubal infertility, the increasing number of men with poor semen quality led to the development of a variety of sperm preparation techniques. These techniques generally fall into four categories: (i) simple dilution and washing, (ii) sperm migration,

(iii) density gradient centrifugation, and (iv) filtration or adherence. Regardless of the technique, the objective of sperm preparation is to recover an enriched population of motile and functionally competent spermatozoa while eliminating dead spermatozoa and other cells, including bacteria and leukocytes. The technique should also minimize damage to the spermatozoa and eliminate decapacitation factors and toxic substances such as reactive oxygen species (ROS). Some of these techniques as well as their advantages and disadvantages are presented here.

SPERM COLLECTION

Ejaculation

The semen specimen should be collected by masturbation and the ejaculate produced into a sterile glass or disposable plastic jar that has been checked for sperm toxicity. As soon as the seminal plasma has liquefied, the specimen should be analyzed according to the WHO guidelines (6) and prepared for sperm isolation. A second semen specimen may be requested if the semen specimen on the day of IVF is of very poor quality (7). When liquefaction is delayed or the specimen is especially viscous, drawing the sample through a 21 gage needle into a syringe may help break up viscous globules. For men who are unable to collect semen by masturbation, nontoxic condoms are commercially available; guidelines for their proper use should be strictly abided by patient and laboratory personnel. Ordinary contraceptive condoms must not be used (even those without spermicide) because of their sperm toxicity. Coitus inter-ruptus is also not recommended because of the risk of incomplete recovery and potential iatrogenic contamination of the ejaculate.

Semen may be collected from men who are unable to achieve erection, emission, or ejaculation because of neurological or psychogenic problems by electroejaculation using direct vibratory stimulation of the penis or electrical stimulation of the prostate. Ejaculates from spinal cord injured patients will frequently have high sperm concentrations, decreased motility, and red blood cell contamination. Sperm may also be recovered from the urine of patients whose ejaculation is retrograde into the bladder. It is advisable that these patients be prescribed stomach-acid buffering medications to make the urine pH more hospitable for sperm.

Surgical

The collection of epididymal and/or testicular spermatozoa requires an office or outpatient surgical procedure. Epididymal spermatozoa can be retrieved either by microsurgery or by percutaneous needle puncture. As the typical indication for epididymal aspiration is obstructive azoospermia rather than testicular dysfunction, it is not uncommon for relatively large

quantities of sperm to be obtained and subsequently used for IVF, or even intrauterine insemination (IUI), and any excess sperm may be frozen for future use. Depending on operator skill, epididymal aspirates can be obtained with minimal red blood cell and non-germ cell contamination, making the isolation and selection of motile sperm quite easy. If large numbers of epididymal spermatozoa are obtained, then density gradient centrifugation (see below) is an effective method for preparing those spermatozoa for subsequent use.

Testicular spermatozoa can be retrieved by open biopsy (with or without microdissection) or by percutaneous needle biopsy. Testicular specimens are contaminated invariably with large amounts of red blood cells and testicular tissues; additional steps are needed to isolate a clean preparation of spermatozoa. In order to free the seminiferous tubule-bound spermatozoa, it is necessary to use either enzymatic (collagenase) or mechanical methods. For the latter, testicular tissues in supportive culture medium is macerated using glass cover slips until a fine slurry of dissociated tissues is produced, and the resulting suspension can then be processed for therapeutic use. Excess testicular spermatozoa obtained in this manner can be frozen for future use in order to avoid further surgeries. Testicular spermatozoa can also be obtained from a needle biopsy, although only a small amount of tissue is usually retrieved and the resulting sperm yield is proportionately low.

SPERM PREPARATION METHODS

Simple Washing and Dilution

The sperm preparation method used for the first IVF cases involved dilution of the semen with culture medium (usually at 2–10 times the volume) and separation of the spermatozoa by centrifugation. After removal of the supernatant, the pellet is resuspended in another aliquot of culture medium. Repeat centrifugation, usually two or three times in total, is often used to ensure removal of contaminating seminal plasma. The centrifugation is usually performed at 200–300 g and it should certainly be performed at centrifugal forces less than 800 g (8). Advantages of this method are that it is the simplest and the least expensive to perform. One disadvantage of this technique is nonviable, and immotile spermatozoa as well as any leukocytes, squamous epithelial cells, or non-cellular debris that contaminated the original semen sample will still be present in the washed sample. Another disadvantage is the concern about potential damage caused by centrifugation.

Aitken and Clarkson (9) reported that techniques involving the repeated centrifugation of unselected populations of human spermatozoa generate cell suspensions with significantly reduced motility. Moreover, these detrimental effects of centrifugation were associated with a sudden burst of ROS produced by a discrete subpopulation of cells characterized

by significantly diminished motility and fertilizing capacity. The ROSs were found to impair the functional competence of normal spermatozoa in the same suspension, reflected in impaired capacity for sperm–oocyte fusion. It has also been shown that ROS can cause DNA damage in human spermatozoa when exposed for time periods consistent with clinical sperm preparation techniques for ICSI or IVF (10). Thus, sperm preparation techniques that involve a washing step in which semen is diluted with culture medium and centrifuged have mostly been abandoned for alternative techniques such as direct swim-up from semen or density gradient centrifugation.

Sperm Migration

Motile spermatozoa separate themselves from seminal plasma in vivo by actively migrating through cervical mucus in the female reproductive tract. There are a variety of sperm preparation techniques that involve migration of spermatozoa, and the element common and prerequisite to all these techniques is the self-propelled movement of spermatozoa.

Swim-Up from Washed Pellet

The swim-up of spermatozoa from a washed pellet technique was originally described by Mahadevan and Baker (11) and it is still a standard method for patients with normozoospermia and female infertility (12). The procedure involves dilution and centrifugation (repeated two to three times) of a semen specimen to separate spermatozoa from seminal plasma. The pellet of spermatozoa formed after the final centrifugation can either be left intact or gently resuspended in the small residual volume of supernatant in the bottom of the centrifugal tube. Swim-up from an intact sperm pellet requires that centrifugation speeds be such that the final pellet is loosely compacted. This can be verified by gently and slowly tilting the test tube and observing whether the pellet tilts as well. Each laboratory should determine the centrifugation time and speed that will afford this attribute. If one chooses to resuspend the sperm pellet, then extreme care must be taken to ensure that no mixing occurs when overlaying the non-compacted pellet with culture medium. If mixing occurs, then the final aspirated supernatant (containing sperm for subsequent use) can be contaminated with immotile sperm, debris, and non-germ cells. This latter technical problem is less of an issue when the sperm pellet is left intact. Regardless of whether an intact or disrupted sperm pellet is used, culture medium is layered over the pellet and the tube is incubated at 37°C for 30–60 minutes to allow the spermatozoa to swim up from the pellet.

As with all techniques involving the mixing of spermatozoa with medium, it is important to choose a culture medium that is buffered appropriately for the atmosphere in which the technique takes place. Therefore, if the incubator atmosphere is the same as the laboratory and the temperature

is 37°C, then the medium should be buffered with HEPES or a similar buffer, and the caps of the swim-up tubes should be tightly closed. If the incubator atmosphere is 5–6% CO_2 and the temperature is 37°C, then the medium is best buffered with sodium bicarbonate or a similar buffer, and the caps of the test tubes should be loose. Adherence to the aforementioned will ensure culture pH that is compatible with sperm survival.

To facilitate the release of motile spermatozoa from the sperm pellet, the test tube may be placed at 45°, thereby increasing the surface area interface between the sperm pellet and the culture medium. Alternatively, aliquots of the resuspended pellet may be placed in 4-well dishes before culture medium is layered over each aliquot. The use of 4-well dishes will also increase the interface between the pellet and the culture medium (13). Evidence that sperm have successfully swim-up into the overlaying culture medium is reflected by an increase in turbidity. If the culture medium appears clear, then more time may be needed to allow spermatozoa the opportunity to swim out of the pellet. After the incubation, the upper layer of culture medium containing spermatozoa is carefully aspirated without disrupting the interface and transferred to a clean test tube from which concentration, motility, and morphology can be assessed.

Advantages of the swim-up from washed pellet method include the recovery of a high percentage of motile sperm and the absence of other cells and debris. Another advantage of this technique is that it consistently produces suspensions of spermatozoa with increased swimming velocity and more normal sperm morphology (14). The swim-up method also results in significant improvement in the rates of acrosome reaction, hypo-osmotic swelling (HOS), and nuclear maturity (15). A disadvantage of the swim-up from washed pellet is the low overall recovery of motile spermatozoa; motile spermatozoa trapped at the bottom of the pellet may never be able to reach the interface with the culture medium. Thus, the efficiency of the technique is based not only on the initial sperm motility in the ejaculate, but also on the size, level of compaction, and exposed surface area of the final pellet. Another disadvantage is the previously discussed concern about potential damage caused by centrifugation of unselected populations of human spermatozoa.

Direct Swim-Up from Semen

A swim-up technique that avoids centrifugation of unselected populations of spermatozoa is the direct swim-up from semen, in which aliquots of liquefied semen are placed underneath a layer of culture medium in either 4-well dishes or a series of test tubes. The interface between the semen layer and the culture medium is increased by placing the tubes at 45° in the incubator. Depending on the initial ejaculate volume, sperm concentration, sperm motility, multiple test tubes, or 4-well dishes may be used to increase the recovery of motile spermatozoa. The interface can often be cleaner when

the liquefied semen is layered under the culture medium with a syringe and needle rather than layering the culture medium over the semen (16).

The test tubes are incubated at 37°C for 30–60 minutes to allow the spermatozoa to swim up from the liquefied semen. Evidence that sperm have successfully swum up into the overlaying culture medium is reflected by an increase in turbidity. If the culture medium appears clear, then more time may be needed to allow spermatozoa the opportunity to swim out of the pellet. After incubation, the upper layer of culture medium in each tube is carefully aspirated and removed to a clean centrifugal tube. The suspension is then centrifuged at 300–600 g for 4–10 minutes after which the supernatant is removed and the pellet resuspended in fresh culture medium to achieve the desired concentration of motile spermatozoa.

Advantages of the direct swim-up method include the recovery of a high percentage of motile sperm and the absence of contaminating dead or immotile spermatozoa, non-germ cells, and debris. In a comparison of four methods for sperm preparation, Ren et al. (17) found that the direct swim-up method provided the best sperm motility. Another advantage is the elimination of the centrifugation step prior to the swim-up, which reduces ROS production by white blood cells and dying spermatozoa. A disadvantage of the direct swim-up from semen is the low recovery of motile spermatozoa.

Migration Sedimentation

The migration-sedimentation method was developed by Tea et al. (18) and it combines the swim-up technique with a sedimentation step in special glass or plastic tubes containing an inner cone. Spermatozoa swim up directly from liquefied semen into the overlying culture medium and subsequently settle gravitationally in the inner cone of the tube. Incubation is usually 60 min at 37°C, after which the medium in the cone is removed and centrifuged at 300g for 5–10 minutes. Sperm count and motility are then determined on the resuspended pellet.

The advantages of the migration-sedimentation method are similar to those of the direct swim-up technique: the migration-sedimentation method is a very gentle separation method and it yields a clean fraction of highly motile spermatozoa. In addition, ROSs are reduced because of the lack of centrifugation prior to sperm migration. The disadvantages of the technique include a very low yield of motile spermatozoa and the requirement for special glass or plastic tubes.

A comparative study by Gabriel and Vawda (19) demonstrated that specimens from fertile males processed using the migration-sedimentation method had the greatest increase in motility and the only increase in morphology versus specimens processed either by filtration (SpermPrep®) or swim-up from washed pellet. Specimens from subfertile males also showed significantly increased sperm motility and morphology when the migration-sedimentation method was used. Gabriel concluded that migration

sedimentation should be the method of choice unless the original sperm count is low.

Sanchez et al. (20) modified the migration-sedimentation method to include an initial centrifugation of the neat semen at 400 g for 10 minutes with the resulting pellet diluted in 500 μL of seminal fluid before being placed under culture medium in special glass tubes and incubated for 2–3 hours at 37°C. After the incubation, the medium in the cone is removed and centrifuged at 300 g for 5–10 minutes. Sperm count and motility are then determined on the resuspended pellet. The extra centrifugation step and the lengthened incubation allowed them to recover a sufficient number of motile spermatozoa even in cases with severe oligozoospermia and/or asthenozoospermia. Using this modified method, Sanchez et al. demonstrated significantly better results in progressive motility, normal morphology, chromatin condensation, and reduction in the percentage of dead spermatozoa when compared with density gradient centrifugation. In spite of the findings of Sanchez et al., one must bear in mind the same cautions when subjecting unselected sperm populations to centrifugation.

Density Gradient Centrifugation

Density gradients may be either continuous or discontinuous although the discontinuous gradients have been used almost exclusively since the late 1980s (21). Discontinuous gradients are usually prepared with two or three layers. Colloidal silica with covalently bound silane molecules is probably the most common density gradient material currently used for clinical IVF and andrology. PureSperm® (NidaCOn International AB, Göteborg, Sweden), Isolate® (Irvine Scientific, Santa Ana, California, U.S.A.), IxaPrep (MediCult, Copenhagen, Denmark), and Enhance® (Conception Technologies, San Diego, California, U.S.A.) are examples of silane-coated silica particle solutions that can be used for discontinuous gradients. These products are made isosmotic by the inclusion of polysucrose; they have very low toxicity, are nonirritating, and are approved for human in vivo use. As with any product, it is important to follow the manufacturer's recommendation for proper use and application.

In the discontinuous density gradient method, the ejaculate is placed on top of the density gradient medium and is centrifuged at 300–400 g for 15–30 minutes. As the density gradient medium is a colloid rather than a solution, it has low viscosity and it does not retard the sedimentation of spermatozoa due to centrifugation (21). Highly motile spermatozoa move actively in the direction of the sedimentation gradient and can penetrate the boundary faster than poorly motile or immotile spermatozoa (12). Thus, the soft pellet at the bottom is enriched for highly motile spermatozoa. The pellet is washed with culture medium and centrifuged at 200 g for 4–10 minutes. The wash and centrifugation is then repeated to ensure removal

of contaminating density gradient medium. The final pellet is resuspended in culture medium so that concentration and motility can be determined.

Density gradient centrifugation usually results in a clean fraction of highly motile spermatozoa. As the whole volume of the ejaculate is used in density gradient centrifugation (as it is in the swim-up techniques), it yields a significantly higher total number of motile spermatozoa and it can be used for patients with varying degrees of suboptimal semen parameters (e.g., oligozoospermia and asthenozoospermia). Other advantages of density gradient centrifugation include the elimination of leukocytes and the significant reduction of ROS (12). Additionally, Nicholson et al. (22) demonstrated that centrifugation through one brand of silane-coated silica particles (PureSperm) efficiently reduces bacterial contamination. Hammadeh et al. (23) reported that another advantage of the density gradient method is the recovery of a higher percentage of morphologically normal spermatozoa than found in conventional swim-up or glass wool filtration. The technique has also been shown to yield sperm populations with better DNA quality and chromatin packaging (24,25). Further, preliminary reports suggest that specimens known to be contaminated with sexually transmissible viruses can effectively be "cleaned up" using density gradient centrifugation and the isolated spermatozoa can be used for therapy with exceptionally low risk for horizontal disease transmission (26). One disadvantage of density gradient centrifugation is that the density gradient medium is a bit more expensive than either of the swim-up techniques.

Adherence—Filtration

These methods are based on the phenomenon that dead and moribund spermatozoa are extremely sticky and will attach to glass surfaces even in the presence of relatively high concentrations of protein (16).

Glass Wool Filtration

In this method, motile spermatozoa are separated from immotile spermatozoa by means of densely packed glass wool fibers. The principle of this technique involves both the self-propelled movement of the spermatozoa and the filtration effect of the glass wool. The method initially employed vertical Pasteur pipettes filled with glass wool fibers on to which the ejaculate was placed and allowed to filter by gravity (27). The method has evolved such that in a current variation (28), the filter is created by placing 30 mg of pre-cleaned glass wool microfibers in the barrel of a 3 mL disposable syringe and gently packing it down using the syringe plunger (minus its rubber tip). The syringe is suspended vertically in a 15 mL centrifuge tube and rinsed several times with culture medium to remove any loose wool fibers prior to filtration. Meanwhile, the ejaculate is washed with an equal volume of culture medium, pipetted into 15 mL centrifuge tubes (no more than

3 mL/tube), and centrifuged at 300 g for 3 minutes. Each resulting pellet is resuspended in 1 mL of culture medium, and centrifuged again at 300 g for 3 minutes. The pellet in one tube is resuspended with 300 μL of culture medium, and this single supernatant is sequentially added to resuspend the sperm pellet in any remaining tubes (the total volume should not exceed 400 μL). The washed sperm suspension is gently pipetted over the pre-wet glass wool column and then allowed to filter by gravity into a clean 15 mL centrifugal tube. When the dripping stops, 100 μL of culture medium is added to the filter and allowed to drip through. The filter is removed and the filtrate can be assessed for sperm concentration and motility.

The success of this method is related to the kind of glass wool used—the chemical nature of the glass, the surface structure and charge of the glass wool, and the thickness of the glass wool fibers. Glass wool from Manville Fiber Glass Corporation (Denver, CO) or SpermFertil® columns from Mello (Holzhausen, Germany) has been tested extensively in clinical practice (12). Glass wool filtration and two-layer, discontinuous density gradient centrifugation resulted in an average recovery of 50–70% of the progressively motile and about 50% of the HOS-positive spermatozoa (29). Additionally, glass wool filtration tended to be more successful than density gradient centrifugation when the ejaculates were asthenozoospermic or had an abnormal HOS test. After processing, the activity of the zona lysing enzyme acrosin increased approximately two- to threefold, but no significant improvement in the percentage of normal sperm forms occurred. Glass wool filtration was also more effective in removing non-motile and HOS-negative spermatozoa than density gradient centrifugation when the percentage of these types of spermatozoa in the ejaculate is high.

This method can use the whole volume of the ejaculate and thus yield a significantly higher total number of motile spermatozoa, which means it can be used for patients with oligozoospermia and/or asthenozoospermic. It is also possible to prepare motile spermatozoa from patients with retrograde ejaculation (12). Another advantage of glass wool filtration is the elimination of up to 90% of the leukocytes present in the ejaculate (30). As leukocytes are a major producer of ROS, elimination of a majority of leukocytes should significantly reduce ROS. Finally, glass wool filtration was also found to yield a significantly higher percentage of chromatin-condensed spermatozoa than swim-up or density gradient centrifugation (30). Disadvantages of the glass wool filtration method include the added expense of the glass wool and a filtrate that is not as clean as it is with other sperm preparation methods because remnants of debris may still be present.

Sephadex Columns

Sperm separation using Sephadex beads is another filtration method (31), and a kit based on this principle (SpermPrep) is commercially available (Fertility Technologies, Inc.). Basically, liquefied semen is diluted with

culture medium and centrifuged at 400 g for 6 minutes. The supernatant is discarded and the sperm pellet resuspended in culture medium to a concentration of 100×10^6 sperm/mL. One milliliter of the washed semen is placed in the filter column containing hydrated filtration beads and mixed gently. The bottom cap is removed from the filter column and fluid is allowed to filter for 15 minutes. The filtrate is centrifuged at 400 g for 6 minutes and resuspended in 1 mL of culture medium before being assessed for concentration, motility, and morphology.

In a comparative study, the yield of spermatozoa post-processing was highest with SpermPrep than with swim-up or migration sedimentation in both fertile and subfertile men (19), and for that reason the authors recommended that specimens with a lower than normal sperm count but normal motility and morphology should be processed with SpermPrep. Disadvantages of Sephadex bead filtration include the added expense of the kit and a filtrate that is not as clean as it is with other sperm preparation methods because remnants of debris may still be present. In addition, the prefiltration centrifugation step might generate ROS.

POST-SEPARATION TREATMENT OF SPERMATOZOA

Improvement of Motility and Sperm Function

Pentoxifylline

The use of methylxanthine derivatives such as pentoxifylline for the stimulation of sperm functions, especially motility, is well known. Pentoxifylline is a nonspecific inhibitor of phosphodiesterase that has stimulatory effects on sperm motility and motion characteristics like sperm velocity or hyperactivity. The stimulatory effect is attributed to increased intracellular levels of cAMP via inhibition of its breakdown by cAMP phosphodiesterase. Pentoxifylline is also reported to enhance the acrosome reaction (32) presumably due to the increasing levels of cAMP. The results of pentoxifylline treatment in assisted reproduction are equivocal. Depending on the conditions, especially the time of stimulation relative to the capacitative state of the spermatozoa and the concentration of pentoxifylline in the medium, overstimulation can result in a premature acrosome reaction (12). Thus, pentoxifylline tends to be used on a limited basis in IVF programs and some programs choose to use pentoxifylline only in the preparation of epididymal and testicular sperm for assisted IVF.

Spermatozoa retrieved from the testis have not experienced the maturation-inducing influence(s) afforded during epididymal transport, and therefore, are in a different physiologic state than epididymal or ejaculated spermatozoa. Treatment of immotile or very poorly motile fresh or

cryopreserved testicular spermatozoa with pentoxifylline very frequently simulates some form of motion, whether it is twitching, nonprogressive motility, or progressive motility. The goal in any ICSI procedure is to use spermatozoa that are viable, and motion is the best indicator ensuring both a functional (protective) plasma membrane and patent metabolic processes. The combination of these two attributes lends greater assurance that the DNA has not been made more vulnerable to the deleterious effects of ROS.

Platelet-Activating Factor

Platelet-activating factor (PAF) is a biologically active phospholipid thought to be a cellular mediator in reproduction that has been found in spermatozoa of many different species, including human (33). PAF has been reported to have positive effects on motility, capacitation, acrosome reaction, and oocyte penetration (34,35), and these stimulatory actions on sperm function can be inhibited by PAF antagonists (36). Although the molecular mechanism of action has yet to be fully elucidated, the positive effect of PAF on sperm function has led to its use in assisted reproduction. Roudebush et al. (37) reported that pregnancy rates in IUI cycles were significantly increased after the spermatozoa from normozoospermic males were prepared with a medium containing PAF.

Detection of Viability

Sperm motility is an important indicator of viability, especially when performing ICSI. In the absence of native or stimulated sperm motility, the assessment of viability becomes critical. There is a simple vitality test based on the semi-permeability of the intact and physiologically functional plasma membrane which causes spermatozoa to swell under hypo-osmotic conditions, when an influx of water results in an expansion of cell volume (6). This vitality test is known as the HOS test.

The HOS test can be used for specimens where the spermatozoa are all immotile. When setting up a dish for the ICSI procedure, a small (5 μL) drop of HOS solution is placed near the PVP drop and two extra drops of culture medium are placed nearby. A small volume of sperm suspension is placed in one of the extra drops. When spermatozoa are located, they are picked up in the ICSI micropipette and placed in the HOS solution. Immediately after contact with the hypo-osmotic medium, the tails of some spermatozoa will begin to coil or swell. Tail swelling or curling indicates that the spermatozoon is undergoing hypo-osmotic stress, the plasma membrane is functional, and the cell is still viable. The spermatozoon is then picked up in the ICSI micropipette and placed in the other extra drop of medium in order to wash off excess hypo-osmotic medium from both the micropipette and the spermatozoon. The spermatozoon is then placed in the PVP drop in order to proceed with ICSI.

SUMMARY

The choice and application of the appropriate sperm preparation technique can be a major contributor in influencing whether a patient will become pregnant. Ejaculates from subfertile males very frequently contain or have the potential for producing ROS, which are known to compromise sperm function and damage DNA. Therefore, it is imperative that the laboratory technologist selects a technique that will directly separate functional and highly motile spermatozoa from all that remains. Although it might be argued that density gradient centrifugation is the most effective method, not all laboratories may have access to the equipment and/or resources needed to perform the technique. Thus, depending on the initial sperm parameters, the direct swim-up from semen can safely and effectively be used and the post-swim-up centrifugation step may be omitted.

Although every specimen is considered valuable, oftentimes the specimens being handled are precious and/or expected to serve as a resource for many attempts at paternity. Thus, one must be even more selective when choosing a sperm-preparation technique. For example, a male undergoing medical therapy may be producing his last sperm-containing ejaculate, so optimization in the total number of motile sperm recovered is necessary. Further, due care is advised when processing an operatively obtained specimen, as repeat surgery exposes the patient to additional (and unnecessary) risk.

After reading this piece, one may ask: What kind of techniques might be on the horizon? Current technologies have afforded new knowledge about sperm biology. Expression of membrane proteins is not only a reflection of properly functioning spermatogenesis and spermiogenesis, but also sperm maturation. This characteristic may be exploited by using techniques that isolate cells based on the ionic charge of expressed membrane proteins (i.e., electrophoretically) or by using reversible binding techniques that involve cell function.

REFERENCES

1. Austin CR. Observations on the penetration of sperm into the mammalian egg. Aust J Scr Res 1951; 4(series B):581.
2. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 1951; 18:697.
3. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. The Physiology of Reproduction. 2nd ed. New York: Raven Press, 1994:189.
4. Rogers BJ, Perreault SD, Bentwood BJ, McCarville C, Hale RW, Soderdahl DW. Variability in the human–hamster in vitro assay for fertility evaluation. Fertil Steril 1983; 39:204.
5. Kanwar KC, Yanagimachi R, Lopata A. Effects of human seminal plasma on fertilizing capacity of human spermatozoa. Fertil Steril 1979; 31:321.

6. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction. 4th ed. Cambridge: Cambridge University Press, 1999:29.
7. Barash A, Lurie S, Weissman A, Insler V. Comparison of sperm parameters, in vitro fertilization results, and subsequent pregnancy rates using sequential ejaculates, collected two hours apart, from oligoasthenozoospermic men. Fertil Steril 1995; 64:1008.
8. Jeulin C, Serres C, Jouannet P. The effects of centrifugation, various synthetic media and temperature on the motility and vitality of human spermatozoa. Reprod Nutr Dev 1982; 22:81.
9. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 1988; 9:367.
10. Lopes S, Jurisicova A, Sun J-G, Casper RF. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 1998; 13:896.
11. Mahadevan M, Baker G. Assessment and preparation of semen for in vitro fertilization. In: Wood C, Trounson A, eds. Clinical In Vitro Fertilization. Berlin: Springer, 1984:83.
12. Henkel RR, Schill W-B. Sperm preparation for ART. Reprod Biol Endocrinol 2003; 1:108.
13. Bongso A. Handbook on Blastocyst Culture. Department of Obstetrics and Gynaecology, National University of Singapore, 1999:26.
14. Oehninger S, Acosta R, Morshedi M, Philput C, Swanson RJ, Acosta AA. Relationship between morphology and motion characteristics of human spermatozoa in semen and in the swim-up sperm fractions. J Androl 1990; 11:446.
15. Erel CE, Senturk LM, Irez T, et al. Sperm-preparation techniques for men with normal and abnormal semen analysis. J Reprod Med 2000; 45:917.
16. Mortimer D, Mortimer ST. Methods of sperm preparation for assisted reproduction. Ann Acad Med 1992; 21:517.
17. Ren S-S, Sun G-H, Ku C-H, Chen D-C, Wu G-J. Comparison of four methods for sperm preparation for IUI. Arch Androl 2004; 50:139.
18. Tea NT, Jondet M, Scholler R. A migration-gravity sedimentation method for collecting motile spermatozoa from human semen. In: Harrison RF, Bonner J, Thompson W, eds. In Vitro Fertilization, Embryo Transfer and Early Pregnancy. Lancaster: MTP Press Ltd, 1984:117.
19. Gabriel LK, Vawda AI. Preparation of human sperm for assisted conception: a comparative study. Arch Androl 1993; 30:1.
20. Sanchez R, Stalf T, Khanaga O, Turley H, Gips H, Schill W-B. Sperm selection methods for intracytoplasmic sperm injection (ICSI) in andrological patients. J Assist Reprod Genet 1996; 13:228.
21. Mortimer D. Sperm preparation methods. J Androl 2000; 21:357.
22. Nicholson CM, Abramsson LA, Holm SE, Bjurulf E. Bacterial contamination and sperm recovery after semen preparation by density gradient centrifugation using silane-coated silica particles at different g forces. Hum Reprod 2000; 15:662.
23. Hammadeh ME, Kühnen A, Amer AS, Rosenbaum P, Schmidt W. Comparison of sperm preparation methods: effect on chromatin and morphology recovery

rates and their consequences on the clinical outcome after in vitro fertilization embryo transfer. Int J Androl 2001; 24:360.
24. Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. Hum Reprod 2001; 16:2160.
25. Morrell JM, Moffatt O, Sakkas D, et al. Reduced senescence and retained nuclear DNA integrity in human spermatozoa prepared by density gradient centrifugation. J Assist Reprod Genet 2004; 21:217.
26. Politch JA, Xu C, Tucker L, Anderson DJ. Separation of human immunodeficiency virus type 1 from motile sperm by the double tube gradient method versus other methods. Fertil Steril 2004; 81:440.
27. Paulson JD, Polakoski KL. A glass wool column procedure for removing extraneous material from the human ejaculate. Fertil Steril 1977; 28:178.
28. Jeyendran RS, Van der Ven HH, Perez-Peleaz M, Al-Hasani S, Diedrich K. Separation of viable spermatozoa by standardized glass wool column. In: Ratnam SS, Teoh E-S, Ng S-C, eds. Vitro Fertilisation. Carnforth, UK: Parthenon Publishing, 1987:49.
29. Rhemrev J, Jeyendran RS, Vermeiden JPW, Zaneveld LJD. Human sperm selection by glass wool filtration and two-layer, discontinuous Percoll gradient centrifugation. Fertil Steril 1989; 51:685.
30. Henkel R, Ichikawa T, Sanchez R, Miska W, Ohmori H, Schill W-B. Differentiation of ejaculates showing reactive oxygen species production by spermatozoa or leukocytes. Andrologia 1997; 29:295.
31. Drobnis EZ, Zhong CQ, Overstreet JW. Separation of cryopreserved human semen using Sephadex columns, washing or Percoll gradients. J Androl 1991; 12:201.
32. Tesarik J, Mendoza C, Carrera A. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reaction in human sperm. Fertil Steril 1992; 58:1185.
33. Minhas BS, Roudebush WE, Ricker DD, Dodson MG. The presence of platelet-activating factor-like activity in human spermatozoa. Fertil Steril 1991; 55:372.
34. Sengoku K, Tamate K, Takaoka Y, Ishikawa M. Effects of platelet-activating factor on human sperm function. Hum Reprod 1993; 8:1443.
35. Krausz C, Gervasi G, Forti G, Baldi E. Effect of platelet-activating factor on motility and acrosome reaction of human spermatozoa. Hum Reprod 1994; 9:471.
36. Cheminade C, Gautier V, Hichami A, Allaume P, Le Lannou D, Legrand AB. 1-*O*-Alkylgycerols improve boar sperm motility and fertility. Biol Reprod 2002; 66:421.
37. Roudebush WE, Telede AA, Kert HI, Mitchell-Leef D, Elsner CW, Massey JB. Platelet-activating factor significantly enhances intrauterine insemination pregnancy rates in non-male factor infertility. Fertil Steril 2004; 81:52.

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Assisted Fertilization

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INTRODUCTION

Since the birth of Louise Brown, the first test tube baby, in 1978 (1), in vitro fertilization (IVF) has become a well-established treatment procedure for certain types of infertility—including long-standing infertility due to tubal disease, endometriosis, unexplained infertility, or infertility involving a male factor. However, it became obvious that certain couples with severe male-factor infertility could not be helped by conventional IVF. Extremely low sperm counts, impaired motility, and poor morphology represent the main causes of failed fertilization in conventional IVF. To tackle this problem, several procedures of assisted fertilization based on micromanipulation of oocytes and spermatozoa have been established. These strategies have culminated in intracytoplasmic sperm injection (ICSI), where a single spermatozoon is directly injected into the ooplasma. In 1992, our group reported the first human pregnancies and births after replacement of embryos generated by this novel procedure of assisted fertilization (2). Since then, the number of worldwide centers offering ICSI has increased tremendously, as has the number of treatment cycles per year (3). Because of the widespread application of ICSI as the ultimate and only option for successful treatment of severe male infertility due to impaired testicular function or obstruction of the excretory ducts, concern about its efficacy and safety is appropriate.

This chapter surveys the current status of ICSI, emphasizing patient selection for ICSI, gamete handling prior to microinjection, the ICSI procedure and outcome parameters of fertilization, and embryo cleavage after ICSI. Furthermore, outcome and children's health after IVF and ICSI will be summarized including pregnancy complications, major malformations, and possible causes of adverse outcome.

INTRACYTOPLASMIC SPERM INJECTION

History of ICSI

Extremely low sperm counts, impaired motility, and abnormal morphology represent the main causes of failed fertilization in conventional IVF. Today, ICSI is the ultimate option to treat these cases of severe male-factor infertility. One single viable spermatozoon, preferably of good morphology, is selected by the embryologist and injected in each oocyte available.

ICSI is based on micromanipulation of oocytes and spermatozoa. Initially, partial zona dissection (PZD) was established to facilitate sperm penetration (4–7). The barrier to fertilization represented by the zona pellucida was disrupted mechanically so that the inseminated sperm cells obtained direct access to the perivitelline space of the oocyte. Subzonal insemination (SUZI) represented the next step in micromanipulation techniques (8–11). SUZI enabled the immediate delivery of several motile sperm cells into the perivitelline space by means of an injection pipette. ICSI is even more invasive because a single spermatozoon is directly injected into the ooplasma, thereby crossing not only the zona pellucida but also the oolemma. ICSI had been first used successfully to obtain live offspring in rabbits and cattle (12), and a preclinical evaluation was reported by the Norfolk group (13). The first human pregnancies and births resulting from this novel assisted-fertilization procedure were reported in 1992 (2). Thereafter, ICSI was revealed to be superior to SUZI in terms of oocyte fertilization rate (14–17), number of embryos produced, and embryo implantation rate (14–17). As a result, ICSI has been used successfully worldwide to treat infertility due to severe oligo-astheno-teratozoospermia, or azoospermia caused by impaired testicular function or obstructed excretory ducts (18,19).

Since the first publication describing the ICSI procedure, minor modifications contributed to reduced rates of oocyte degeneration, oocyte activation (one-pronuclear), and abnormal fertilization (three-pronuclear). Hyaluronidase may be responsible for oocyte activation; therefore, the concentration used during oocyte denudation and the exposure time of oocytes to the enzyme have been reduced (20). The moment of denudation relative to oocyte pick-up (immediately or four hours later) does not influence the ICSI results (21). The orientation of the polar body during injection does, however, influence embryo quality (22). Motile sperm cells are selected and

immobilized prior to injection (23). Cytoplasm aspiration to ensure oolemma rupture is critical to the success of the ICSI procedure because the method of rupture has been correlated with oocyte degeneration (22). Furthermore, the morphology of the injected spermatozoon is related to the fertilization outcome of the procedure as well as to the pregnancy outcome (24).

Indications for ICSI

Before the era of ICSI, attempts were made to modify and refine conventional IVF to achieve increased rates of conception in cases of male-factor infertility. Today, ICSI has clearly overshadowed the use of modified IVF procedures (including high insemination concentration) for the treatment of severe male-factor infertility. ICSI requires only one spermatozoon with a functional genome and centrosome for the fertilization of each oocyte. Indications for ICSI are not restricted to impaired morphology of the spermatozoa, but also include low sperm counts and impaired kinetic quality of the sperm cells. ICSI can also be used with spermatozoa from the epididymis or testis when there is an obstruction in the excretory ducts. Azoospermia caused by testicular failure can be treated by ICSI if enough spermatozoa can be retrieved in testicular tissue samples. Table 1 gives an overview of the current indications for ICSI.

Table 1 Current Indications for Intracytoplasmic Sperm Injection

Ejaculated spermatozoa
Oligozoospermia
Asthenozoospermia (caveat for 100% immotile spermatozoa)
Teratozoospermia ($\leq$4% normal morphology using strict criteria-caveat for globozoospermia)
High titers of antisperm antibodies
Repeated fertilization failure after conventional IVF
Autoconserved frozen sperm from cancer patients in remission
Ejaculatory disorders (e.g., electroejaculation, retrograde ejaculation)
Epididymal spermatozoa
Congenital bilateral absence of the vas deferens
Young syndrome
Failed vaso-epididymostomy
Failed vasovasostomy
Obstruction of both ejaculatory ducts
Testicular spermatozoa
All indications for epididymal sperm
Failure of epididymal sperm recovery because of fibrosis
Azoospermia caused by testicular failure (maturation arrest, germ-cell aplasia)
Necrozoospermia

Abbreviation: IVF, in vitro fertilization.

ICSI with ejaculated spermatozoa can be used successfully in patients with fertilization failures after conventional IVF and also in patients with too few morphologically normal and progressive motile spermatozoa present in the ejaculate (<500,000). High fertilization and pregnancy rates can be obtained when a motile spermatozoon is injected. Injection of only immotile or probably non-vital spermatozoa results in lower fertilization rates (25). In cases where only non-vital sperm cells are present in the ejaculate, the use of testicular sperm is indicated (26). Other semen parameters, such as concentration, morphology (except for globozoospermia) (27), and high titers of antisperm antibodies (28) do not influence the success rates of ICSI (25). Successful ICSI has also been described for patients with acrosomeless spermatozoa (29,30).

Any form of infertility due to obstruction of the excretory ducts can be treated by ICSI with spermatozoa microsurgically recovered from either the epididymis (31–33) or the testis (34–36). Obstructive azoospermia can result from congenital bilateral absence of the vas deferens, failed vasectomy reversal, or vaso-epididymostomy. When no motile spermatozoa can be retrieved from the epididymis due to epididymal fibrosis, testicular spermatozoa can be isolated from a testicular biopsy specimen.

Testicular biopsy has also proven to be useful in some cases of non-obstructive azoospermia (37–40). In patients with severely impaired testicular function due to (incomplete) germ-cell aplasia (Sertoli-cell-only syndrome), hypo-spermatogenesis, or incomplete maturation arrest, spermatozoa may be recovered, sometimes only, after taking multiple biopsies. Testicular sperm recovery may not always be successful in all azoospermic patients. Cryopreservation of supernumerary spermatozoa recovered from the epididymis (41) or the testis (42) is an important issue because microinjection of cryo-thawed sperm cells can avoid repeated surgery in future ICSI cycles.

The ICSI procedure cannot be carried out in approximately 3% of the scheduled cycles. The most common causes for cancellation are either no cumulus–oocyte complexes or metaphase II oocytes are available, or no spermatozoa are found in testicular biopsies of patients with non-obstructive azoospermia.

Gamete Handling Prior to ICSI

A successful ICSI program depends on ovarian stimulation, which is essentially similar to methods used for conventional IVF. Current ovarian stimulation regimens use a combination of gonadotropin-releasing hormone (GnRH) agonists or antagonists, human menopausal gonadotropin (hMG), or recombinant follicle-stimulating hormone (recFSH), and human chorionic gonadotropin (hCG), which allows the retrieval of a high number of cumulus–oocyte complexes (43,44). Administration of GnRH agonists

allows for pituitary down-regulation to occur before the initiation of exogenous FSH. Gonadotropin preparations or recFSH are administered to stimulate multiple follicle development. Ovulation is usually induced with hCG (10,000 IU), which is administered when the serum estradiol level exceeds 1000 pg/mL, and when at least three follicles of 18 mm or more in diameter are observed on ultrasound examination. The optimal time for ultrasound-guided transvaginal oocyte aspiration is 36 hours after hCG administration. On average, 11 cumulus–oocyte complexes per cycle can be retrieved (45). After cumulus and corona cell removal, approximately nine metaphase II oocytes per cycle are available for microinjection (45).

Although hCG may be used for luteal-phase supplementation, exogenous progesterone (administered intravaginally) is frequently applied in an attempt to avoid the risk of hCG stimulation of remaining growing follicles.

More recently, the clinical introduction of GnRH antagonists (46,47) allows a powerful and immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous LH and FSH (48). By making optimal use of endogenous FSH, the amount of exogenous FSH required for follicular growth could be substantially reduced. A rapid recovery of pituitary LH and FSH release after cessation of GnRH antagonist administration might permit the abandonment of additional luteal-phase support.

Fertilization by means of micromanipulation requires denudation of oocytes (i.e., removal of the surrounding cumulus and corona cells). This strategy allows not only precise injection of the oocytes, but also the assessment of their maturity, which is of critical importance for ICSI. Cumulus and corona cells are removed using a combination of enzymatic and mechanical procedures (20). Both the enzyme concentration and the duration of the exposure to the enzyme should be limited because they can result in parthenogenetic activation of the oocytes (49). Microscopic observations of the denuded oocytes include assessment of the zona pellucida and the oocyte, and the presence or absence of a germinal vesicle (GV) or a first polar body. Ninety-five percent of the retrieved cumulus–oocyte complexes usually contain an intact oocyte. The remaining 5% represent empty zonae, cracked zonae, or morphologically abnormal oocytes.

Figure 1 shows different stages of oocyte nuclear maturity. On average, 3.9% of the intact oocytes are at the metaphase I stage, having undergone breakdown of the GV but not extrusion of the first polar body (45). Approximately 10.3% of the intact oocytes are at the GV stage, and about 85.8% of them are in the metaphase II stage, showing the presence of the first polar body (45). ICSI is only carried out on metaphase II oocytes because only such oocytes have reached the haploid state and, thus, can be fertilized normally. Frequently, metaphase I oocytes achieve meiosis after a few hours in vitro and are available for ICSI on the day of oocyte retrieval. Despite lower fertilization rates (52.7 vs. 70.8%), injection of matured

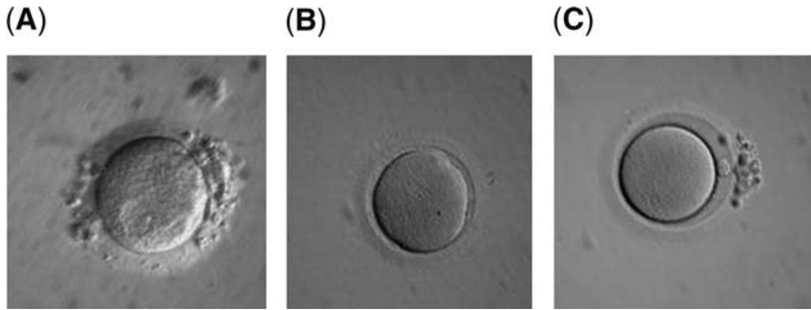

Figure 1 Oocyte maturity after cumulus and corona cell removal. (**A**) A germinal vesicle (GV) stage oocyte is recognized by the presence of a typical GV. (**B**) Oocytes that have undergone GV breakdown but not yet extruded the first polar body are called metaphase I oocytes. (**C**) A typical metaphase II oocyte displays the presence of a first polar body, which indicates that the oocyte is mature and has reached the haploid state. Only metaphase II oocytes are submitted to intracytoplasmic sperm injection.

metaphase I oocytes results in embryos of similar quality to metaphase II oocytes at the moment of oocyte retrieval (50). Denuded and rinsed oocytes are incubated until the time of microinjection.

For microinjection, spermatozoa from three different origins are processed: ejaculated sperm and surgically retrieved sperm from the epididymis or the testis. For all three categories, ICSI in combination with sperm cryopreservation is currently used successfully (41,51,52). All patients selected for ICSI with ejaculated semen undergo a preliminary semen assessment prior to the treatment cycle to verify whether enough spermatozoa (preferably motile) are present to perform ICSI.

Routinely, sperm samples for ICSI are processed by density-gradient centrifugation [using silane-coated silica particle colloid solutions (53)], enriching the number of motile and morphologically normal sperm cells needed for assisted reproduction. Only in cases of extreme oligozoospermia (i.e., when gradient centrifugation results in an insufficient yield of sperm cells for ICSI) is simple washing of the sperm sample performed to reduce the loss of sperm cells for injection. Immediate injection of the oocytes is then indicated because sperm cells lose their initial motility and often die when the sample is simply washed. This consequence can be ascribed to the presence of reactive oxygen species and other damaging substances (54,55).

During microsurgical epididymal sperm aspiration (56,57), several sperm fractions are collected into separate tubes. Sperm fractions with similar concentration and motility are pooled, and a density-gradient centrifugation is performed. Micro-droplets of the re-suspended pellet are placed in separate medium droplets adjacent to a central polyvinylpyrrolidone

(PVP) droplet in the injection dish. This facilitates the search for and the selection of single motile spermatozoa. Spermatozoa are collected using a testicular sperm extraction (TESE) pipette, which is larger in diameter than an injection pipette (outer diameter 8–10 μm instead of 6–7 μm). The spermatozoa are then transferred to the PVP droplet and immobilized prior to injection. Whenever possible, some of the freshly recovered sperm should be frozen for later use in subsequent ICSI cycles, thereby avoiding repeated surgical procedures (56).

Testicular biopsy specimens, usually obtained by means of surgical excisional biopsy (35), are shredded into small pieces with sterile microscope slides (58) on the heated stage of a stereomicroscope. The presence of spermatozoa is assessed with an inverted microscope, which determines whether the surgical procedure can be stopped or whether extra biopsy pieces need to be taken. The pieces of biopsy tissue are removed, and the medium is centrifuged at 300*g* for 5 minutes. The pellet is then re-suspended for the ICSI procedure. Single motile spermatozoa are collected in a manner similar to the way epididymal sperm are, using separate medium droplets that contain fractions of the testicular sperm suspension. If no sperm cells can be found, the tissue pieces can be treated with red blood cell lysis buffer (59) or an enzymatic collagen digestion medium (60). Lysis of excess red blood cells may facilitate the search for sperm cells, and the use of enzymes may result in the recovery of otherwise inaccessible sperm cells that are initially attached to the tissue. It is well known that it is not always possible to retrieve testicular spermatozoa from biopsy specimens in patients with non-obstructive azoospermia due to germ-cell aplasia or maturation arrest (61). In cases where testicular spermatozoa are retrieved under local anesthesia by means of the fine-needle aspiration approach (62), the aspirated fractions are immediately collected in the injection dish. No further sample processing, except collecting the single motile spermatozoa with a microneedle (TESE pipette), is needed prior to ICSI.

ICSI Procedure

For the ICSI procedure itself, an inverted microscope equipped with micromanipulators and microinjectors should be available (63). Magnification capability of 200× and 400× is a prerequisite for precise procedures such as ICSI. A heating stage on the inverted microscope maintains the temperature at 37°C. Ambient temperature control is of vital importance for the survival of oocytes, which are very sensitive to a decrease in temperature that can cause irreversible damage to the meiotic spindle (64). The micromanipulators allow three-dimensional manipulation (coarse and fine movements) of the holding and injection pipette on the left- and right-hand side, respectively. The microinjectors are used to either fix or release the oocyte with the holding pipette, or to aspirate and inject a spermatozoon with the injection

pipette. The injectors can be air-filled or filled with mineral oil. A micrometer controls the plunger. The whole setup is placed on a vibration-proof table to avoid possible interfering motion. Several companies supply micro-tools for holding and injection; however, some centers still prepare their own micro-tools, which demands extra effort, time, and specialized equipment (63).

The ICSI procedure involves the injection of a single motile spermatozoon into the oocyte. The procedure is carried out in a plastic microinjection dish containing micro-droplets covered with mineral oil. A fraction ($\pm 1\ \mu L$) of the sperm suspension is added to the periphery of the central PVP droplet. Separate medium droplets are used in cases of epididymal or testicular sperm. The oocytes, denuded from their surrounding cumulus and corona cells, are placed in the eight surrounding medium droplets. The viscous character of the PVP solution slows down the motility of sperm cells, thereby facilitating the manipulation. It also allows better control of the fluid in the injection needle and prevents sperm cells from sticking to the pipette.

During ICSI, the following steps can be distinguished: selection and immobilization of a viable sperm cell, correct positioning of the oocyte prior to injection, and rupture of the oolemma prior to the release of the sperm cell into the oocyte.

Figure 2 illustrates the whole injection procedure. In the injection pipette, which is filled with PVP, a single living, morphologically normal spermatozoon is aspirated. Viability is evidenced by the motility of the sperm cell, even if it is only a slight twitching of the tail. The sperm cell is then released in a perpendicular position to the injection pipette, which facilitates immobilization. Immobilization of a sperm cell involves rubbing the tail with the pipette against the bottom of the dish, which results in a breakage at one point, preferably below the midpiece. Immobilization of spermatozoa has been proven to be important for oocyte activation, which is achieved by release of sperm cytosolic factors via the ruptured membrane. Increased fertilization rates with ICSI have been reported for the following aggressive damage to the sperm tail plasma membrane (23,65).

After immobilization, the sperm cell is again aspirated (now tail-first) to allow the injection of a minimal volume of medium together with the sperm cell. The oocyte is held in position by means of minimal suction by the holding pipette. The polar body is located at the six o′clock position, which avoids damage to the spindle (22). Experiments in our laboratory using Hoechst dye-stained oocytes for microinjection clearly showed no interference with the spindle if oocytes are injected with the polar body at the six o′clock position. If both the holding pipette and the oocyte are in perfect focus, the injection needle containing the immobilized sperm cell near the tip can be introduced in the equatorial plane of the oocyte at the three o′clock position. Permanent focus of the injection pipette tip ensures that the needle remains in the equatorial plane of the oocyte. Passing through the zona pellucida is fairly easy and achieved by simply advancing the

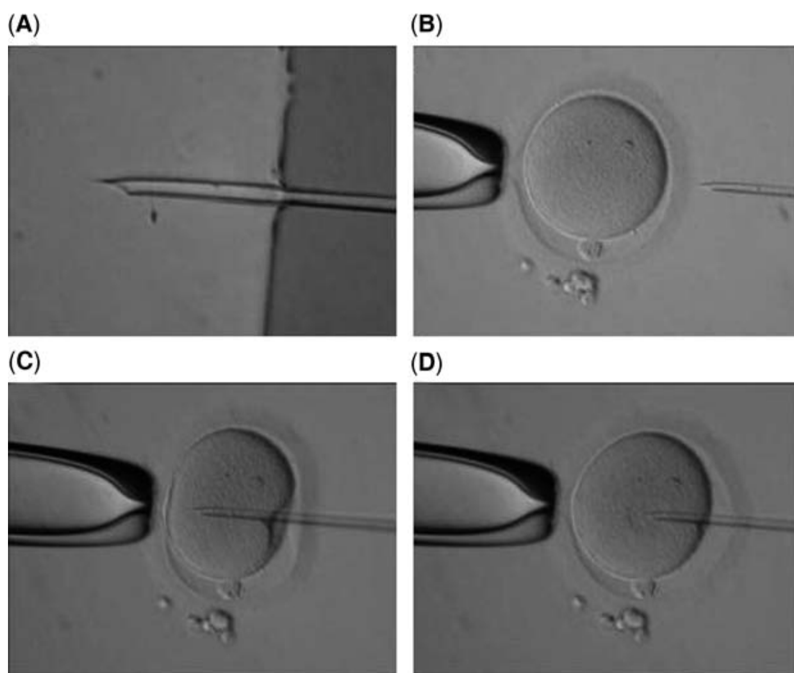

Figure 2 Intracytoplasmic sperm injection procedure. (**A**) A single motile spermatozoon is selected and immobilized by pressing its tail between the microneedle and the bottom of the dish. The sperm cell is then aspirated tail-first into the injection pipette. (**B**) Using the holding pipette, the mature oocyte is fixed with the polar body at the 6 o′clock position. The sperm cell is brought to the tip of the injection pipette. (**C**) The injection pipette is introduced at the 3 o′clock position and rupture of the oolemma is ascertained by slight suction. Then the sperm cell is delivered into the oocyte with a minimal volume of medium; afterwards, the pipette can be carefully withdrawn. (**D**) A single sperm cell can be appreciated in the center of the ooplasma.

injection pipette. In contrast, the oolemma is not always immediately pierced by simple injection of the needle and often minimal suction needs to be applied. The ooplasm then enters into the injection pipette, and sudden acceleration of the flow indicates membrane rupture. The aspiration is immediately stopped, and the sperm cell is then slowly released into the oocyte with a minimal volume of medium and the pipette can be withdrawn carefully.

Different patterns of oolemma breakage have been described, depending on whether the ooplasm breaks during insertion of the pipette, whether slight or stronger aspiration of the ooplasrm is needed, or whether breakage of the oolemma in another place has to be attempted (22,66). Immediate rupture of the oolemma without any aspiration has been associated with lower oocyte survival rates (22,66).

Fertilization and Embryo Cleavage After ICSI

After the injection procedure, oocytes are rinsed and cultured in micro-droplets covered with lightweight paraffin oil. The conditions are similar to those employed for IVF inseminated oocytes: the oocytes are kept at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Injected oocytes are examined for integrity and fertilization about 16–18 hours after ICSI (67). An average damage rate of approximately 9% of the injected oocytes can be expected, irrespective of the origin of the sperm used (45). Oocytes are considered normally fertilized when two individualized or fragmented polar bodies are present together with two clearly visible pronuclei (2-PN) that contain nucleoli (Fig. 3).

The fertilization rate after ICSI is usually expressed per number of injected oocytes and ranges from 57% to 67% according to the sperm origin (45). As shown in Figure 3, abnormal fertilization may occur, reflected by one-pronuclear (1-PN) oocytes (about 3% of the injected oocytes) (45). These oocytes are likely to be parthenogenetically activated as a result of mechanical or chemical factors (68,69). The occasional finding of three-pronuclear (3-PN) oocytes (about 4%) (45) after injection of a single spermatozoon into the ooplasm is probably caused by failure of extrusion of the second polar body at the time of fertilization (69). Neither type of embryo resulting from 1-PN to 3-PN oocytes is transferred to patients.

Post-fertilization, about 90% of 2-PN oocytes obtained by ICSI enter cleavage, resulting in multicellular embryos. Cleavage characteristics of the fertilized oocytes are evaluated daily. Normally developing, good-quality embryos reach the four-cell and eight-cell stage, respectively, on day 2 and in the morning of day 3 postmicroinjection (Fig. 4). Numbers and sizes of blastomeres and the presence of anucleate cytoplasmic fragments are

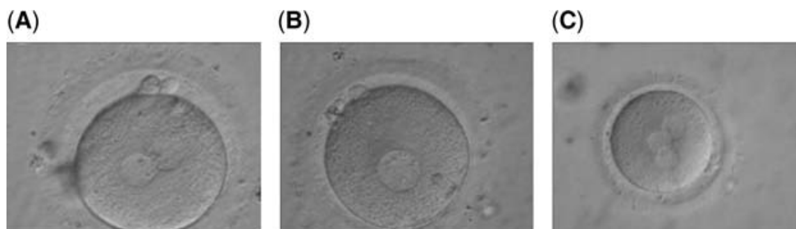

Figure 3 Fertilization outcome after intracytoplasmic sperm injection. (**A**) Oocytes are considered normally fertilized when two individualized or fragmented polar bodies are present together with two clearly visible pronuclei (2-PN) that contain nucleoli. (**B**) Abnormal fertilization may occur as one pronuclear (1-PN) oocyte, probably due to parthenogenic activation. (**C**) The occasional finding of three pronuclear (3-PN) oocytes after injection of a single spermatozoon into the ooplasm is probably caused by non-extrusion of the second polar body at the time of fertilization.

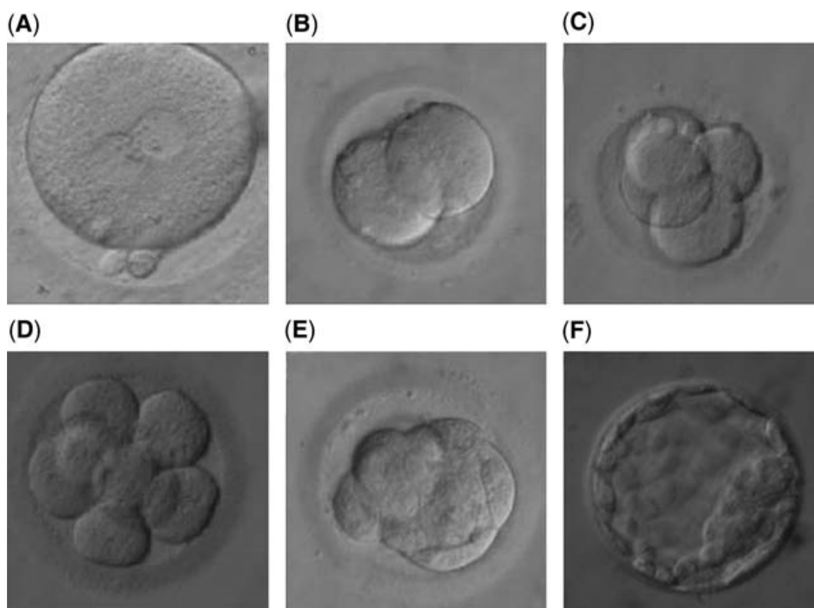

Figure 4 Embryo cleavage after intracytoplasmic sperm injection. Only embryos resulting from normally fertilized oocytes (**A**) will be transferred to patients. Embryo cleavage is evaluated daily. Two-cell embryos (**B**), four-cell embryos (**C**), and eight-cell embryos (**D**) are usually obtained on day 1 (late afternoon), on day 2, and in the morning of day 3, respectively. The blastomere number is recorded and the embryos are scored accordingly to equality of size of the blastomeres and the presence of anucleate cytoplasmic fragments. On day 4 (sometimes already on day 3), a certain degree of compaction can be observed (**E**). For blastocyst (**F**) scoring, the classification system introduced by Gardner and Schoolcraft is used. Embryo transfer is usually done on day 3 (eight-cell stage) or day 5 (blastocyst stage). *Source*: From Refs. 70 and 75.

recorded. The cleaving embryos are scored according to equality of size of the blastomeres and proportion of anucleate fragments (70). Type A (excellent quality) embryos do not contain anuclear fragments. Type B (good quality) embryos have a maximum of 20% of the volume of the embryo filled with anucleate fragments. In type C (fair quality) embryos, anucleate fragments represent 21% to 50% of the volume of the embryo. Type D (poor quality) embryos have anucleate fragments present in more than 50% of the volume of the embryos. These embryos cannot be used for transfer to the patients. Embryos in the former three categories (type A, B, and C) are eligible for transfer.

Nowadays, most centers perform embryo transfers on day 3 or day 5 after oocyte retrieval. At that time, the embryos are expected to be at the

eight-cell stage. Because the embryonic genome is fully activated after the eight-cell stage (71), it may indeed be beneficial to evaluate embryos at least until after the transition from maternal to embryonic genome, making it possible to identify those embryos with a better developmental potential. The number of embryos transferred depends on the age of the woman and on the rank of trial. In women under 37 years of age who are undergoing a first or second ICSI attempt, preference is given to transfer of only two excellent or good-quality embryos. In other cases, three or more embryos may be placed into the uterus. Higher pregnancy rates can be obtained when elective transfer of two or three embryos is possible (45,72). However, the number of embryos transferred should be limited in order to avoid multiple pregnancies.

Today, commercially available sequential culture media allows the culture of human embryos up to the blastocyst stage (day 5 or 6) (73). On day 4 (sometimes already on day 3), a certain degree of compaction can be observed. Compaction in the mammalian pre-embryo is a fundamental event that leads to the formation of the trophectoderm, the inner cell mass and the blastocoele. Full compaction (16–32-cell stage) is followed by immediate cavitation and blastocoele expansion (74). For blastocyst scoring, the classification system introduced by Gardner and Schoolcraft can be used (75). A distinction between early and expanded blastocysts is made, and the latter category is further scored, according to the quality of the inner cell mass and the trophectoderm. The possibility of prolonged human embryo culture allows for day 5 or blastocyst transfers. Preferably, expanded blastocysts with a cohesive trophectoderm and a clear inner cell mass are transferred. Possible advantages of blastocyst transfer are better embryo selection and better synchronization between embryo and endometrium, which may result in higher implantation rates per blastocyst transferred (76,77). This in turn would allow transfer of fewer embryos, thereby decreasing the number of multiple pregnancies (78).

OUTCOME AND CHILDREN'S HEALTH

For all forms of assisted reproductive technology (ART), the most important outcome parameter is the health of the children born after ART. The birth of a healthy singleton birth has to be considered as the most important outcome parameter after ART (79). Even after several decades of ART practice, one has to realize that it is impossible to give an answer with regards to risks for pregnancy and birth complications for ovarian stimulation in view of timed intercourse and intrauterine insemination. Only in IVF and ICSI have enough data been collected to provide a valid estimation of the risks. Even then, there are limitations in the study design of IVF and ICSI follow-up which make it impossible to estimate whether it is the ART procedure or the underlying infertility of the treated couples that influence

the outcome (80). Several aspects of ART outcome will be reviewed: pregnancy complications, major malformations, possible reasons for adverse outcome as well as the increase of multiple ART pregnancies.

Pregnancy Complications

Perinatal outcome of singletons born after IVF have been recently assessed in a meta-analysis (81). The study compared for the period 1978–2002, a cohort of 12,283 IVF and ICSI singletons to a control cohort of 1.9 million spontaneously conceived singletons, matched for maternal age and parity. In comparison with spontaneous conceptions, IVF and ICSI pregnancies were associated with significantly higher odds of each of the perinatal outcome parameters studied: perinatal mortality, pre-term delivery, low birth weight, very low birth weight, and small gestational age. In the ART singletons, the prevalence was higher for early pre-term delivery, spontaneous pre-term delivery, placenta praevia, gestational diabetes, pre-eclampsia, and neonatal intensive care admission. IVF patients must be counseled about these adverse perinatal outcomes, and obstetricians should manage these pregnancies as high risk. A systematic review by Helmerhorst et al. of perinatal outcome of singletons and twins after ART confirmed the data for singletons of the Jackson et al. meta-analysis (82). The systematic review comprises 25 studies (17 with matched and 8 with non-matched controls) published between 1985 and 2002. For singletons, the review indicated a significant increased relative risk for very pre-term (<32 weeks) and pre-term (<37 weeks) deliveries. The relative risks were also increased for very low birth weight (<1500 g), low birth weight (<2500 g), small for gestational age, caesarean section, admission to neonatal intensive care unit, and perinatal mortality. Matched and non-matched studies gave similar results. For matched and non-matched studies of twin gestations, the above-mentioned outcome parameters were similar between ART and control pregnancies. Perinatal mortality was lower in assisted conception twins compared to natural conception twins.

Major Malformations

The question whether there is an increased risk for major congenital malformations after IVF or ICSI was recently reviewed in two meta-analysis (83,84). The meta-analysis by Hansen et al. (83) indicated an overall increase after IVF and ICSI. This was also the case when only singletons, IVF children or ICSI children were analyzed separately. The pooled odds ratio risk for major birth defects was 1.32 (confidence interval 1.20–1.45). A meta-analysis by Lie et al. (85) compared major malformations in 5935 ICSI children to 13,086 conventional IVF children. The relative risk for a major malformation after ICSI was 1.2 (95% CI 0.97–1.28). The meta-analysis by Rimm et al. (84) confirmed the higher risk of major malformations in IVF

and ICSI children in comparison to spontaneously conceived children. There was no significant difference in the risk when IVF and ICSI were compared.

A multicentric cohort study (86) of the physical health of 5-yr-old children conceived after ICSI ($n = 540$), IVF ($n = 538$), or natural conception ($n = 437$) indicated that in comparison with natural conception the odds ratio for major malformations was 2.77 (CI 1.41–5.46) for ICSI and 1.80 (CI 0.85–3.81) for IVF children. Socio-demographic factors did not affect these results. The higher rate observed in the ICSI group was partially due to an excess in the (boys') urogenital system. In addition, IVF and ICSI children were more likely than naturally conceived children to have had a significant childhood illness, to have had a surgical operation, to require medical therapy, and to be admitted to hospital. It will be important to continue monitoring of these children. As reported by Ludwig (87), there are major gaps in valid data to assess major malformations after the different ART procedures as compared with spontaneously conceived children from fertile couples. This is the case for ovarian stimulation and intrauterine insemination. The major limitations of studies on major malformation rates include the absence of a control cohort, the use of historical controls with unclear definitions, the data collection in the control, and study group as well as in the definitions of the term "major malformation" (88).

Possible Causes of Adverse Outcome

As indicated by Ludwig (89), factors may be involved at different steps of the ART treatment that may lead to an increased risk of adverse outcome.

The genetics of the male and female partners may influence the outcome. It has been well established that there are more constitutional abnormal karyotypes in infertile males and females. Several studies also indicated that abnormal sperm have more chromosomal abnormalities. In a cohort of 1298 ICSI parents seen for genetic counseling, it was concluded that there was an increased genetic risk for 557 of these children (45). This increased risk was due to maternal or paternal age, chromosomal aberrations, monogenic or multifactorial disease, and consanguinity. Slightly less than 5% of infertile males and 1.5% of tested females had an abnormal karyotype. With regard to fetal karyotypes after ART, there are only systematic data available for ICSI (90). Results on 1586 fetal karyotypes indicated an increased risk related to the chromosomal anomalies in the parents. The majority of cases (17 out of 22) were paternally inherited. There were significantly more de novo anomalies (1.6%), but the absolute risk is low. More anomalies were observed when sperm concentration was $<20 \times 10^6$ sperm per ml and when sperm motility was impaired. Although the ICSI procedure is much more invasive than conventional IVF, there is no difference in outcome between ICSI and IVF (83–85,91). This contrasts with the observations of the abnormalities in the fertilization process after ICSI as compared

to IVF in rhesus monkeys (92). In theory, all manipulations of gametes and embryos such as gamete preparation and manipulation, in vitro culture, blastomere biopsy, and assisted hatching could influence the constitution of the embryo and ultimately the health of fetuses and children. Efforts should be pursued to establish multinational registries to collect data on the offspring, as has been done by the ESHRE Consortium on Preimplantation Genetic Diagnosis (93). Strict quality management in the IVF laboratory (such as strict temperature control) is indicated because of its influence on outcome. In recent literature, case reports and case–control studies have been published on the occurrence of imprinting disorders in ART children. There are cases of Angelman syndrome (94) and Beckwith–Wiedeman syndrome (95,96). The absolute risk for these imprinting disorders in ART remains low, and so far the reason for an increased risk of imprinting errors remains unknown. As outlined by Buck Louis et al.(80), a major drawback of all outcome studies is that the control group is fertile, and the study group is unfertile. It would therefore be indicated that a comparison should be made between ART conceptions and spontaneous conceptions in a subfertile population. With regard to pregnancy complications, a study from the United Kingdom (97) indicated that there is an increased incidence of abruptio placentae, pre-eclampsia, and Caesarean section in couples with idiopathic infertility compared to fertile couples whether conception was spontaneous or after infertility treatment. Similar observations were made in the United States, Denmark, and Sweden (98–101). The question of why the risks are increased remains unanswered: is it due to in vitro conditions or to the infertility status per se? To assess the contribution of in vitro handling, risk assessment for malformations may be done comparing ovarian stimulation alone or in combination with intrauterine insemination.

Multiple Pregnancies After ART

There is increasing evidence that the major outcome risk after all forms of ART is the occurrence of multiple pregnancies and births. This is the case for ovulation induction, ovarian hyperstimulation with or without intrauterine insemination, and IVF or ICSI (102). For IVF–ICSI, the number of children born has been estimated to be about two million. This positive observation is overshadowed by the fact that at least half of these children are not from singleton pregnancies. The occurrence of multiple IVF–ICSI pregnancies and births is, of course, due to the placement of more than one embryo. There is extensive evidence that multiple pregnancies and births generate more problems not only during pregnancy and delivery, but also later in life (102). Therefore, prevention of multiple ART gestation should be considered as a top priority for all infertility treatments. It is obvious that the practice of single embryo transfer may be the answer to this epidemic of multiple births.

REFERENCES

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978; 2(8085):366.
2. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992; 340(8810):17–18.
3. Tarlatzis BC, Bili H. Intracytoplasmic sperm injection: survey of world results. Ann NY Acad Sci 2000; 900:336–344.
4. Cohen J, Malter H, Wright G, et al. Partial zona dissection of human oocytes when failure of zona pellucida penetration is anticipated. Hum Reprod 1989; 4(4):435–442.
5. Cohen J, Malter H, Fehilly C, et al. Implantation of embryos after partial opening of oocyte zona pellucida to facilitate sperm penetration. Lancet 1988; 2(8603):162.
6. Cohen J, Talansky BE, Malter H, et al. Microsurgical fertilization and teratozoospermia. Hum Reprod 1991; 6(1):118–123.
7. Tucker MJ, Bishop FM, Cohen J, et al. Routine application of partial zona dissection for male factor infertility. Hum Reprod 1991; 6(5):676–681.
8. Laws-King A, Trounson A, Sathananthan H, et al. Fertilization of human oocytes by microinjection of a single spermatozoon under the zona pellucida. Fertil Steril 1987; 48(4):637–642.
9. Ng SC, Bongso A, Ratnam SS, et al. Pregnancy after transfer of sperm under zona. Lancet 1988; 2(8614):790.
10. Bongso TA, Sathananthan AH, Wong PC, et al. Human fertilization by microinjection of immotile spermatozoa. Hum Reprod 1989; 4(2):175–179.
11. Palermo G, Joris H, Devroey P, et al. Induction of acrosome reaction in human spermatozoa used for subzonal insemination. Hum Reprod 1992; 7(2):248–254.
12. Iritani A. Micromanipulation of gametes for in vitro assisted fertilization. Mol Reprod Dev 1991; 28(2):199–207.
13. Lanzendorf SE, Maloney MK, Veeck LL, et al. A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. Fertil Steril 1988; 49(5):835–842.
14. Palermo G, Joris H, Derde MP, et al. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. Fertil Steril 1993; 59(4):826–835.
15. Van Steirteghem A, Liu J, Nagy Z, et al. Use of assisted fertilization. Hum Reprod 1993; 8(11):1784–1785.
16. Van Steirteghem AC, Liu J, Joris H, et al. Higher success rate by intracytoplasmic sperm injection than by subzonal insemination: report of a second series of 300 consecutive treatment cycles. Hum Reprod 1993; 8(7):1055–1060.
17. Van Steirteghem AC, Nagy Z, Joris H, et al. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum Reprod 1993; 8(7): 1061–1066.
18. Society for Assisted Reproductive Technology; American Society for Reproductive Medicine. Assisted reproductive technology in the United States: 2000 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. Fertil Steril 2004; 81(5):1207–1220.

19. Andersen AN, Gianaroli L, Felberbaum R, et al. The European IVF-monitoring programme (EIM), European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2001. Results generated from European registers by ESHRE. Hum Reprod 2005; 20(5):1158–1176.
20. Van de Velde H, Nagy ZP, Joris H, et al. Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. Hum Reprod 1997; 12(10):2246–2250.
21. Van de Velde H, De Vos A, Joris H, et al. Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. Hum Reprod 1998; 13(11):3160–3164.
22. Nagy ZP, Liu J, Joris H, et al. The influence of the site of sperm deposition and mode of oolemma breakage at intracytoplasmic sperm injection on fertilization and embryo development rates. Hum Reprod 1995; 10(12):3171–3177.
23. Palermo GD, Schlegel PN, Colombero LT, et al. Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. Hum Reprod 1996; 11(5):1023–1029.
24. De Vos A, Van De Velde H, Joris H, et al. Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 2003; 79(1):42–48.
25. Nagy ZP, Liu J, Joris H, et al. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. Hum Reprod 1995; 10(5):1123–1129.
26. Tournaye H, Liu J, Nagy Z, et al. The use of testicular sperm for intracytoplasmic sperm injection in patients with necrozoospermia. Fertil Steril 1996; 66(2): 331–334.
27. Liu J, Nagy Z, Joris H, et al. Successful fertilization and establishment of pregnancies after intracytoplasmic sperm injection in patients with globozoospermia. Hum Reprod 1995; 10(3):626–629.
28. Nagy ZP, Verheyen G, Liu J et al. Results of 55 intracytoplasmic sperm injection cycles in the treatment of male-immunological infertility. Hum Reprod 1995; 10(7):1775–1780.
29. Lundin K, Sjogren A, Nilsson L, et al. Fertilization and pregnancy after intracytoplasmic microinjection of acrosomeless spermatozoa. Fertil Steril 1994; 62(6):1266–1267.
30. Bourne H, Liu DY, Clarke GN, et al. Normal fertilization and embryo development by intracytoplasmic sperm injection of round-headed acrosomeless sperm. Fertil Steril 1995; 63(6):1329–1332.
31. Silber SJ, Nagy ZP, Liu J, et al. Conventional in vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. Hum Reprod 1994; 9(9):1705–1709.
32. Tournaye H, Devroey P, Liu J, et al. Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of the vas deferens. Fertil Steril 1994; 61(6):1045–1051.
33. Mansour RT, Aboulghar MA, Serour GI, et al. Intracytoplasmic sperm injection using microsurgically retrieved epididymal and testicular sperm. Fertil Steril 1996; 65(3):566–572.

34. Schoysman R, Vanderzwalmen P, Nijs M, et al. Successful fertilization by testicular spermatozoa in an in vitro fertilization programme. Hum Reprod 1993; 8(8):1339–1340.
35. Silber SJ, Van Steirteghem AC, Liu J, et al. High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. Hum Reprod 1995; 10(1):148–152.
36. Devroey P, Liu J, Nagy Z, et al. Normal fertilization of human oocytes after testicular sperm extraction and intracytoplasmic sperm injection. Fertil Steril 1994; 62(3):639–641.
37. Devroey P, Liu J, Nagy Z, et al. Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. Hum Reprod 1995; 10(6):1457–1460.
38. Tournaye H, Camus M, Goossens A, et al. Recent concepts in the management of infertility because of non-obstructive azoospermia. Hum Reprod 1995; 10(suppl 1):115–119.
39. Kahraman S, Ozgur S, Alatas C, et al. Fertility with testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermic men. Hum Reprod 1996; 11(4):756–760.
40. Silber SJ, van Steirteghem A, Nagy Z, Liu, et al. Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. Fertil Steril 1996; 66(1):110–117.
41. Nagy Z, Liu J, Janssenswillen C, et al. Using ejaculated, fresh, and frozen-thawed epididymal and testicular spermatozoa gives rise to comparable results after intracytoplasmic sperm injection. Fertil Steril 1995; 63(4):808–815.
42. Kupker W, Schlegel PN, Al-Hasani S, et al. Use of frozen-thawed testicular sperm for intracytoplasmic sperm injection. Fertil Steril 2000; 73(3):453–458.
43. Diedrich K, Felberbaum R. New approaches to ovarian stimulation. Hum Reprod 1998; 13(suppl 3):1–13.
44. Macklon N, Fauser BC. Regulation of follicle development and novel approaches to ovarian stimulation for IVF. Hum Reprod Update 2000; 6(4):307–312.
45. Bonduelle M, Camus M, De Vos A, et al. Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. Hum Reprod 1999; 14(suppl 1):243–264.
46. Bouchard P, Fauser BC. Gonadotropin-releasing hormone antagonist: new tools vs. old habits. Fertil Steril 2000; 73(1):18–20.
47. Olivennes F, Cunha-Filho JS, Fanchin R, et al. The use of GnRH antagonists in ovarian stimulation. Hum Reprod Update 2002; 8(3):279–290.
48. Hall JE. Gonadotropin-releasing hormone antagonists: effects on the ovarian follicle and corpus luteum. Clin Obstet Gynecol 1993; 36(3):744–752.
49. Pickering SJ, Johnson MH, Braude PR, et al. Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. Hum Reprod 1988; 3(8):978–989.
50. De Vos A, Van de Velde H, Joris H, et al. In vitro matured metaphase-I oocytes have a lower fertilization rate but similar embryo quality as mature metaphase-II oocytes after intracytoplasmic sperm injection. Hum Reprod 1999; 14(7):1859–1863.

51. Gil-Salom M, Romero J, Rubio C, et al. Intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Mol Cell Endocrinol 2000; 169(1–2): 15–19.
52. Wood S, Thomas K, Schnauffer K, et al. Reproductive potential of fresh and cryopreserved epididymal and testicular spermatozoa in consecutive intracytoplasmic sperm injection cycles in the same patients. Fertil Steril 2002; 77(6): 1162–1166.
53. Soderlund B, Lundin K. The use of silane-coated silica particles for density gradient centrifugation in in vitro fertilization. Hum Reprod 2000; 15(4): 857–860.
54. Griveau JF, Le Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. Int J Androl 1997; 20(2):61–69.
55. Ford WC, Whittington K, Williams AC. Reactive oxygen species in human sperm suspensions: production by leukocytes and the generation of NADPH to protect sperm against their effects. Int J Androl 1997; 20(suppl 3):44–49.
56. Devroey P, Silber S, Nagy Z, et al. Ongoing pregnancies and birth after intracytoplasmic sperm injection with frozen-thawed epididymal spermatozoa. Hum Reprod 1995; 10(4):903–906.
57. Silber SJ, Nagy Z, Liu J, et al. The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility. Hum Reprod 1995; 10(8):2031–2043.
58. Verheyen G, De Croo I, Tournaye H, et al. Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. Hum Reprod 1995; 10(11):2956–2959.
59. Nagy ZP, Verheyen G, Tournaye H, et al. An improved treatment procedure for testicular biopsy specimens offers more efficient sperm recovery: case series. Fertil Steril 1997; 68(2):376–379.
60. Crabbe E, Verheyen G, Tournaye H, et al. The use of enzymatic procedures to recover testicular germ cells. Hum Reprod 1997; 12(8):1682–1687.
61. Tournaye H, Verheyen G, Nagy P, et al. Are there any predictive factors for successful testicular sperm recovery in azoospermic patients? Hum Reprod 1997; 12(1):80–86.
62. Tournaye H, Clasen K, Aytoz A, et al. Fine needle aspiration versus open biopsy for testicular sperm recovery: a controlled study in azoospermic patients with normal spermatogenesis. Hum Reprod 1998; 13(4):901–904.
63. Joris H, Nagy Z, Van de Velde H, et al. Intracytoplasmic sperm injection: laboratory set-up and injection procedure. Hum Reprod 1998; 13(suppl 1): 76–86.
64. Pickering SJ, Braude PR, Johnson MH, et al. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. Fertil Steril 1990; 54(1):102–108.
65. Fishel S, Lisi F, Rinaldi L, et al. Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. Hum Reprod 1995; 10(3):497–500.
66. Palermo GD, Alikani M, Bertoli M, et al. Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by intracytoplasmic sperm injection. Hum Reprod 1996, 11(1):172–176.

67. Nagy ZP, Liu J, Joris H, et al. Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. Hum Reprod 1994, 9(9):1743–1748.
68. Sultan KM, Munne S, Palermo GD, et al. Chromosomal status of uni-pronuclear human zygotes following in vitro fertilization and intracytoplasmic sperm injection. Hum Reprod 1995; 10(1):132–136.
69. Staessen C, Van Steirteghem AC. The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in vitro fertilization. Hum Reprod 1997; 12(2): 321–327.
70. Staessen C, Camus M, Khan I, et al. An 18-month survey of infertility treatment by in vitro fertilization, gamete and zygote intrafallopian transfer, and replacement of frozen-thawed embryos. J In Vitro Fert Embryo Transf 1989; 6(1):22–29.
71. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 1988; 332(6163):459–461.
72. Staessen C, Nagy ZP, Liu J, et al. One year's experience with elective transfer of two good quality embryos in the human in vitro fertilization and intracytoplasmic sperm injection programmes. Hum Reprod 1995; 10(12):3305–3312.
73. Gardner DK, Lane M. Culture of viable human blastocysts in defined sequential serum-free media. Hum Reprod 1998; 13(suppl 3):148–159.
74. Hardy K, Warner A, Winston RM, et al. Expression of intercellular junctions during preimplantation development of the human embryo. Mol Hum Reprod 1996; 2(8):621–632.
75. Gardner DK, Lane M, Stevens J, et al. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 2000; 73(6):1155–1158.
76. Olivennes F, Hazout A, Lelaidier C, et al. Four indications for embryo transfer at the blastocyst stage. Hum Reprod 1994; 9(12):2367–2373.
77. Scholtes MC, Zeilmaker GH. A prospective, randomized study of embryo transfer results after 3 or 5 days of embryo culture in in vitro fertilization. Fertil Steril 1996; 65(6):1245–1248.
78. Karaki RZ, Samarraie SS, Younis NA, et al. Blastocyst culture and transfer: a step toward improved in vitro fertilization outcome. Fertil Steril 2002; 77(1):114–118.
79. Wennerholm UB, Bergh C. What is the most relevant standard of success in assisted reproduction? Singleton live births should also include preterm births. Hum Reprod 2004; 19(9):1943–1945.
80. Buck Louis GM, Schisterman EF, Dukic VM, et al. Research hurdles complicating the analysis of infertility treatment and child health. Hum Reprod 2005; 20(1):12–18.
81. Jackson RA, Gibson KA, Wu YW, et al. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. Obstet Gynecol 2004; 103(3):551–563.
82. Helmerhorst FM, Perquin DA, Donker D, et al. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. BMJ 2004; 328(7434):261.

83. Hansen M, Bower C, Milne E, et al. Assisted reproductive technologies and the risk of birth defects-a systematic review. Hum Reprod 2005; 20(2):328–338.
84. Rimm AA, Katayama AC, Diaz M, et al. A meta-analysis of controlled studies comparing major malformation rates in IVF and ICSI infants with naturally conceived children. J Assist Reprod Genet 2004; 21(12):437–443.
85. Lie RT, Lyngstadaas A, Orstavik KH, et al. Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods: a meta-analysis. Int J Epidemiol 2005; 34(3):696–701.
86. Bonduelle M, Wennerholm UB, Loft A, et al. A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. Hum Reprod 2005; 20(2):413–419.
87. Ludwig M. Development of children born after IVF and ICSI. Reprod Biomed Online 2004; 9(1):10–12.
88. Kurinczuk JJ, Bower C. Birth defects in infants conceived by intracytoplasmic sperm injection: an alternative interpretation. BMJ 1997; 315(7118):1260–1265.
89. Ludwig M. Is there an increased risk of malformations after assisted reproductive technologies? RBM Online 2005; 10(3):83–89.
90. Bonduelle M, Van Assche E, Joris H, et al. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod 2002; 17(10):2600–2614.
91. Bonduelle M, Liebaers I, Deketelaere V, et al. Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999). Hum Reprod 2002; 17(3):671–694.
92. Hewitson L, Dominko T, Takahashi D, et al. Unique checkpoints during the first cell cycle of fertilization after intracytoplasmic sperm injection in rhesus monkeys. Nat Med 1999; 5(4):431–433.
93. Sermon K, Moutou C, Harper J, et al. ESHRE PGD Consortium data collection IV: May–December 2001. Hum Reprod 2005; 20(1):19–34.
94. Ludwig M, Katalinic A, Gross S, et al. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. J Med Genet 2005; 42(4):289–291.
95. Chang AS, Moley KH, Wangler, et al. Association between Beckwith–Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. Fertil Steril 2005; 83(2):349–354.
96. Halliday J, Oke K, Breheny S, et al. Beckwith–Wiedemann syndrome and IVF: a case–control study. Am J Hum Genet 2004; 75(3):526–528.
97. Pandian Z, Bhattacharya S, Templeton A. Review of unexplained infertility and obstetric outcome: a 10 year review. Hum Reprod 2001; 16(12):2593–2597.
98. Williams MA, Goldman MB, Mittendorf R, et al. Subfertility and the risk of low birth weight. Fertil Steril 1991; 56(4):668–671.
99. Henriksen TB, Baird DD, Olsen J, et al. Time to pregnancy and preterm delivery. Obstet Gynecol 1997; 89(4):594–599.
100. Basso O, Weinberg CR, Baird DD, et al. Danish National Birth Cohort. Subfecundity as a correlate of preeclampsia: a study within the Danish National Birth Cohort. Am J Epidemiol 2003; 157(3):195–202.

101. Ghazi HA, Spielberger C, Kallen B. Delivery outcome after infertility–a registry study. Fertil Steril 1991; 55(4):726–732.
102. Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment. Lancet 2005; 365(9473):1807–1816.

11

Analysis of Fertilization

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INTRODUCTION

Fertilization is a process whereby two specialized parental cells, gametes, unite to form a new entity the embryo. Even though the process of fertilization is usually considered as completed as soon as the zygote—a new cell resulting from the fusion between the male gamete (spermatozoon) and the female gamete (oocyte)—is ready to enter the first embryonic mitotic division, the exact time point at which fertilization begins is much less clearly defined. The outcome of fertilization, i.e., a viable embryo, depends on a variety of events beginning with the selection of the one spermatozoon which will eventually fuse with the oocyte and culminate in the signaling events that result from the interaction between sperm-derived and oocyte-derived molecules after gamete union which initiates the early embryonic development. It is in this broader sense that the topic of fertilization is dealt with in this chapter.

Aware of the fact that the mere description of the cellular and molecular events underlying fertilization would outreach the space allowed, this biological background is reduced to a strict minimum necessary for the

understanding of clinically relevant fertilization abnormalities, their diagnosis, and treatment. Particular attention is paid to the current possibilities to predict embryo developmental potential by using data obtained by noninvasive methods of fertilization assessment in assisted reproduction treatment (ART) techniques. Possibilities of alleviating fertilization anomalies in couples at risk are discussed.

BIOLOGICAL BASIS OF FERTILIZATION

Biologically speaking, fertilization involves four sequential steps: selection of the spermatozoon that eventually will fertilize, penetration of the fertilizing spermatozoon through the oocyte vestments (which actually also makes part of the selection process), fusion between the sperm and oocyte plasma membranes (which leads to cytoplasmic syngamy), and nuclear syngamy whereby the genomes of both gametes irreversibly blend to form a new embryonic genome.

Sperm Selection

During sexual intercourse, several tens of millions of spermatozoa are deposited in the vagina. From these, only one will fertilize the oocyte in the end. The selection takes place at multiple sequential steps, when the ejaculated spermatozoa have to negotiate environments that—owing to their physical, chemical, or biological properties—act as barriers. The low intravaginal pH is incompatible with sperm survival for extended time periods so that only those spermatozoa that can rapidly enter the less hostile cervical canal remain in play. Cervical mucus slows down sperm progression and this phenomenon is accentuated by the complicated architecture of the cervical canal. Because sexual intercourse and ovulation are not biologically linked in humans, this situation increases the chance of conception by retaining potentially fertilizing spermatozoa in the cervical crypts, thus allowing their progressive release and enabling fertilization during a prolonged time after sperm deposition in the vagina. Obviously, this phenomenon requires a sufficiently long sperm survival and functional competence.

Sperm Penetration Through Oocyte Vestments

Before the fertilizing spermatozoon reaches the oocyte, it has to penetrate two vestments, the cumulus oophorus and the zona pellucida. Sperm passage through the oocyte vestments involves receptor-mediated interactions with molecules present in the vestments, mainly progesterone in the cumulus oophorus and the ZP3 glycoprotein in the zona pellucida (reviewed in Ref. 1). These interactions are needed to prepare the spermatozoon for the final phase of fertilization—this preparation is commonly called sperm capacitation. Sperm capacitation is a necessary prerequisite for the exocytotic

event called acrosome reaction, which is essential to zona pellucida penetration (1). Obviously these steps imply further selective pressure upon spermatozoa, since those sperm cells that lack functional receptors and signal transducing pathways cannot reach the oocyte.

Sperm–Oocyte Fusion and Oocyte Activation

After sperm penetration through the zona pellucida, the sperm plasma membrane fuses with the oocyte plasma membrane in a narrow equatorial region of the head, followed by incorporation of the whole spermatozoon to the oocyte by a phagocytosis-like process (1). All components of the spermatozoon, with the exception of nuclear DNA, are subsequently dismantled in the oocyte cytoplasm.

An immediate consequence of sperm–oocyte fusion is oocyte activation (2). Shortly after the fusion, a particular type of calcium signal is generated in mammalian oocytes. These signals were first described in the mouse (3), but similar signals were later observed in oocytes of all mammalian species, including the human (4,5). The temporal pattern of these signals is characterized by periodically repeated sharp increases (spikes) in free cytoplasmic calcium ion concentration, also referred to as calcium oscillations (Fig. 1). The spatial propagation of these signals takes the form of calcium waves of which the first is triggered at the sperm-fusion site (Fig. 2A). These phenomena are mediated by fluxes of calcium ions which enter the cytosolic compartment along the pre-existing concentration gradients, both from the extracellular space and from the intracellular calcium

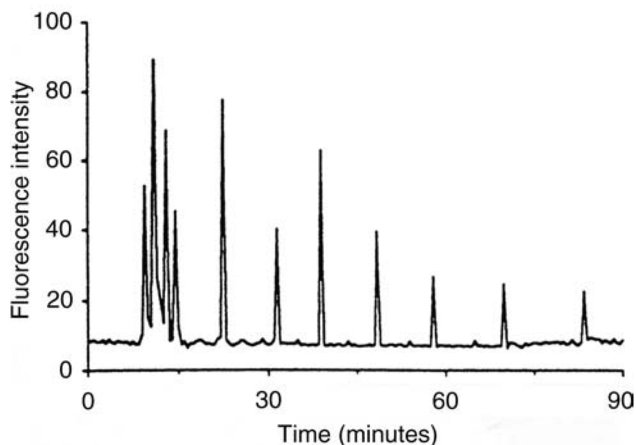

Figure 1 Calcium oscillations, visualized with the use of the intracellular calcium indicator Fluo3, in a living human oocyte fertilized by subzonal insemination. *Source*: From Ref. 5.

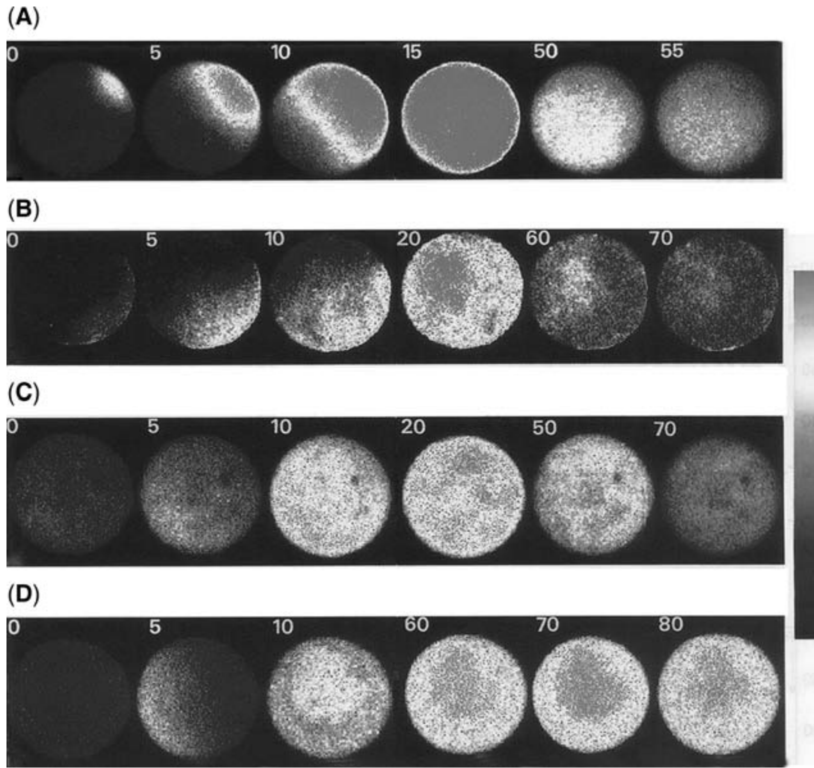

Figure 2 Confocal microscopy images of sperm-induced intracellular free calcium concentration increases, and the effects of protein kinase C(PKC) activation and inhibition. Calcium was visualized by fluorescence microscopy after loading oocytes with intracellular calcium indicator Fluo-3. Differences in fluorescence intensity were converted to color according to the scale bar where the lowest values are coded black. (**A**) The first sperm-induced calcium increase in a control, without any PKC modulation. (**B**) The first sperm-induced calcium increase in an oocyte pretreated with the PKC activator 4β-phorbol 12-myristate 13-acetate before the exposure to spermatozoa. (**C**) The first sperm-induced calcium increase in an oocyte pretreated with the PKC inhibitor chelerythrine before the exposure to spermatozoa. (**D**) One of the ongoing series of calcium increases in an oocyte exposed to the PKC inhibitor chelerythrine after sperm penetration. *Source*: From Ref. 6.

organelles rich in calcium stores, mainly the endoplasmic reticulum (6). The movements of calcium ions between different intracellular calcium-storing organelles and the cytosol are mediated by two types of receptor-gated calcium channels—the inositol-trisphosphate-gated one and the ryanodine-gated one (7)—whose sensitivity to calcium-induced calcium release is modified by an yet poorly defined sperm-borne factor (8) so as to promote

the ability of the oocyte calcium homeostasis machinery (the complex of calcium channels and calcium pumps) to generate calcium waves and calcium oscillations. The steady state required for the maintenance of calcium waves and calcium oscillations is likely to depend on a fine tuning of the phosphorylation status of components of the receptor-gated calcium channels, as these phenomena are disturbed by both activators (Fig. 2B) and inhibitors (Fig. 2C and D) of protein kinase C (PKC) (6).

Oocyte activation triggers the cortical reaction which modifies the properties of the zona pellucida by rendering it resistant to penetration of supernumerary spermatozoa (1). Moreover, it reactivates the cell cycle of the fertilized oocyte by removing substances which maintain the temporary arrest at metaphase of the second meiotic division; this event launches the beginning of the mitotic cell cycle of the newly formed embryo (1).

Pronuclear Development and Nuclear Syngamy

At the time of sperm penetration, the oocyte cell cycle is temporarily arrested in the prolonged metaphase of the second meiotic division. The removal of the cell-cycle-blocking substances, resulting from oocyte activation, will thus release molecular events leading to anaphase, the extrusion of the second polar body and the reformation of an interphase nucleus (female pronucleus). The sperm-derived DNA is more developmentally advanced with regard to oocyte-derived DNA at the time of sperm penetration, but it is not yet ready to respond to oocyte cytoplasmic factors regulating the cell cycle because of its particular organization (packaging), which has been necessary to reduce the sperm nuclear volume. The developmental delay of the oocyte-derived genome with regard to the sperm-derived genome is thus compensated by the delay required for the sperm-derived genome to rearrange. Ideally, these two phenomena are completed almost at the same time.

Sperm nuclear packaging is mediated by a special type of DNA-associated proteins, called protamins, which are different from histones, the proteins associated with DNA in somatic cell and oocyte nuclei. The first molecular modification experienced by the sperm nucleus after its deposition in the oocyte cytoplasm is thus the removal of protamins and their replacement with oocyte-derived histones (1). This step requires previous removal of the sperm nuclear envelope. The assembly of a new nuclear envelope then occurs simultaneously around the sperm-derived and the oocyte-derived nuclear DNA, resulting in the formation of the male and the female pronucleus, respectively.

Both pronuclei undergo rotation-like movements in the oocyte cytoplasm and approach each other progressively until they achieve a close apposition. Concomitantly, the pronuclear chromatin differentiates and nucleolar precursor bodies (NPBs) are formed (9) in a process which

requires an early wave of RNA synthesis (10). The apposition of both pronuclei is mediated by microtubules that are organized with the participation of a sperm-derived centriole. When pronuclear differentiation is complete, the respective nuclear envelopes disassemble, and both the male and the female nuclear materials merge together. This step is called nuclear syngamy. Shortly after nuclear syngamy, the first embryonic cell division (cleavage) begins.

SPECIFIC FEATURES OF FERTILIZATION IN ASSISTED REPRODUCTION

Conventional IVF

As compared with natural fertilization, sperm selection is less stringent in in vitro fertilization (IVF) because the fertilizing spermatozoon does not need to penetrate through the cervical mucus and to make its path along the female genital tract until it meets the oocyte. Hence, the probability of fertilization with less motile and less viable spermatozoa is higher. However, nonprogressively moving and immotile spermatozoa are still excluded as well as spermatozoa with defective function of binding sites for the zona pellucida and oolemma on their plasma membrane and those with abnormal acrosomal function.

Intracytoplasmic Sperm Injection

The fact that sperm entry into the oocyte is entirely independent of both sperm movement and the integrity of the sperm functions required for the zona pellucida binding and penetration for the binding and fusion with the oolemma marks an additional important release of sperm selection stringency. Sperm motility has no more importance, except for that of an easily perceptible marker of sperm viability. The presence and function of sperm surface binding sites and acrosomal enzymes becomes completely irrelevant with regard to the fertilizing ability.

Under these conditions, however, the importance of another set of factors, some of which were not perceived in the conventional IVF era because of their association with other defects that impeded sperm entry to the oocyte, gains importance. Most of these factors are related to success of oocyte activation or failure and to the early development of the zygote (11).

Intracytoplasmic Injection of Immature Male Germ Cells

The success of intracytoplasmic sperm injection (ICSI) in cases of extreme deficiency of sperm movement and morphology (12,13) has prompted attempts at using immature male germ cells for fertilization. These efforts were aimed at offering an ART option to men with azoospermia. Term pregnancies were achieved with epididymal and testicular spermatozoa as

well as with in vivo and in vitro matured spermatids and with secondary spermatocytes (14).

Immaturity of male germ cell nucleus (incomplete chromatin condensation) and cytoplasm (functional inferiority of developmentally relevant cytoplasmic components—centriole and oocyte-activating factor) were suspected to decrease success rates of assisted reproduction with immature male germ cells (15). However, birth rates after fertilization with round spermatids from healthy experimental animals were close to those achieved with spermatozoa (16). Consequently, the relatively low clinical success rates reported after fertilization with round spermatids from infertile men appear to be related to the testicular pathology underlying maturation arrest rather than to germ cell immaturity itself (14).

Fertilization with immature human germ cells often gives rise to mononucleated ova (17). Even though this picture strongly resembles that of artificial oocyte activation without the participation of the male genome, in other cases the single nucleus apparently results from pronuclear fusion (a syngamy nucleus), and the resulting embryos can implant and develop to term (17). This particularity of nuclear syngamy has to be taken into account while assessing fertilization results with immature male germ cells.

NORMAL VS. ABNORMAL FERTILIZATION

Cell Signaling Events

In humans, as all other mammalian species studied so far, normal fertilization is characterized by a particular type of calcium signals consisting of calcium oscillations and calcium waves (refer to previously discussed section "Sperm–Oocyte Fusion and Oocyte Activation"). The same form of calcium signals was observed after conventional IVF (4), subzonal insemination (18), ICSI (5,18), and round spermatid injection (19). Animal experiments have suggested that the quality of the signal encoded by the frequency and amplitude of calcium oscillations is important not only for the immediate success of fertilization, but that it conditions further post-fertilization development during a relatively extended time period, up to the blastocyst stage (20–22).

It is reasonable to suspect that calcium waves and oscillations are beneficial for normal embryonic development in humans, too. It was suggested that truncated calcium signals in human oocytes at fertilization compromise the completion of meiotic division (23) and may cause chromosome nondisjunction leading to aneuploidy (24). Abnormal patterns of fertilization-associated calcium signals can be produced experimentally by exposing oocytes to activators (Fig. 2B) and inhibitors (Fig. 2C and D) of PKC (6). It was noted that experimental modifications of the temporal and spatial patterns of sperm-induced calcium signals in human oocytes often lead to abnormalities of subsequent developmental events, namely the

formation and internal structural differentiation of the male and the female pronucleus (11). Similar abnormalities occasionally occur spontaneously after conventional IVF or ICSI and are particularly frequent in some cases, suggesting the existence of sperm- or oocyte-derived activation abnormalities as the underlying cause.

The Function of Microtubule Organizing Centers

The microtubule organizing center (MTOC) is responsible for the initiation of microtubule organization in the meiotic and mitotic spindles. In humans, as in most other mammalian species, the oocyte MTOCs disappear after fertilization, and the sperm-derived centriole forms the basis for de novo development of the embryonic MTOC (25,26). If the oocyte is fertilized by a spermatozoon with a functionally abnormal centriole, microtubule organization is impaired and the function of the meiotic and mitotic spindles is defective (27,28). This functional abnormality can be revealed by a heterologous ICSI test using bovine oocytes and cytochemical visualization of microtubuli in newly formed asters with the use of antibodies against acetylated- and β-tubulins (28).

Zygote Morphology

Formation and differentiation of pronuclei are the most prominent morphological events taking place in the early postfertilization period. This process was studied in detail by electron microscopic analysis of male pronuclear development in polyspermically penetrated human zona-free oocytes (9). It was found that the assembly of NPBs in the pronuclei was dependent on RNA synthesis (29), and on the activity of ooplasmic factors (30).

Cortical granule exocytosis and redistribution of cytoplasmic organelles (31), in particular those involved in calcium homeostasis (32), are other submicroscopical features of the early post-fertilization development. These events underlie morphological changes that can be observed by light microscopy in living human zygotes. Some of these changes were proposed as markers of zygote developmental potential (refer to subsequent section "Predictive Potential of Zygote Noninvasive Evaluation").

PREDICTIVE POTENTIAL OF ZYGOTE NONINVASIVE EVALUATION

In the late 1990s, it was noted that the timing and regularity of developmental changes in the male and the female pronucleus in human oocytes fertilized in vitro, including pronuclear growth, rotation, and internal differentiation and polarization (Fig. 3), are related to further embryonic development (33–35). Several studies suggested pronuclear zygote scoring systems which were based on the evaluation of the number, size, and

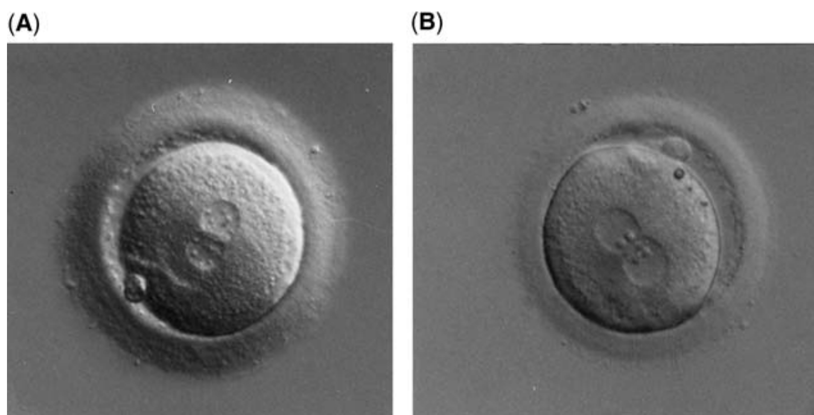

Figure 3 Micrographs showing the size and distribution of nudeolar precursor body (NPBs) in pronuclei of human zygotes at different phases of pronuclear development. (**A**) Relatively early phase of pronuclear development, characterized by a high number of NPB in both pronuclei (only part of them is visible at this focal level). The NPBs are relatively small at this phase and are distributed randomly in the pronuclei. (**B**) Later phase of pronuclear development, characterized by a low number of NPBs in both pronuclei. The NPBs are larger at this phase and show a polarized distribution, with accumulation near that pole of each pronucleus at which this pronucleus makes contact with the other one. (Hoffman modulation contrast, original magnification ×200.). *Source*: From Ref. 35.

distribution of NPBs (36). With the exception of one study (37), all other studies agreed that the developmental potential of preimplantation embryos can be predicted by pronuclear evaluation. In concrete terms, pronuclear morphology was reported to predict cleavage speed and cleaving embryo morphology grade (34,35), the risk of developmental arrest during the first 3 days after fertilization (35,38), the risk of blastomere multinucleation (35), the chance of achieving the blastocyst stage in prolonged culture (39,40), and the risk of chromosomal abnormalities (41–43).

From the clinical point of view, these studies made it possible to make decisions about the selection of embryos to be transferred and of those to be cryopreserved for later use as early as the pronuclear zygote stage. Zygote cryopreservation was reported to give excellent outcomes (44), but it was used relatively rarely for fear of not having enough fresh embryos for transfer because of developmental arrest or abnormal development of those embryos that were allowed to cleave. The possibility of prediction of the quality of preimplantation development on the basis of noninvasive zygote evaluation gave a new impetus to zygote cryopreservation. This was particularly important in countries in which cryopreservation of embryos after the first cleavage division was banned by the law, namely in Germany. In fact,

several German studies reported satisfactory outcomes of assisted reproduction with embryo selection at the zygote stage with the use of pronuclear assessment criteria (45–47).

These observations have underscored the interest in noninvasive zygote evaluation not only as the only guide for embryo selection, as was the case in Germany, but also as part of a combined sequential evaluations at days 1, 2, and 3 after fertilization (48). With the use of a cumulative scoring system, involving post-fertilization day 1, day 2, and day 3 parameters, the efficacy of day 3 embryo transfer was shown to be comparable to that of day 5 blastocyst transfer (49).

ETIOLOGY AND DIAGNOSIS OF ABNORMAL FERTILIZATION

Fertilization abnormalities mostly result from deficiencies of sperm-derived oocyte-activating factor of those of the oocyte cytoplasmic systems that have to generate an adequate response to the sperm factor (also refer to section "Normal Versus Abnormal Fertilization"). In either situation, the signaling events implicated in nuclear and cytoplasmic reprogramming required for the gamete-to-embryo transition are failing. In other cases, fertilization problems are caused by sperm centriole abnormalities which lead to disturbances of the MTOC function which is required for pronuclear apposition, nuclear syngamy, and the subsequent cleavage divisions.

Many of the conditions underlying abnormal fertilization can now be analyzed, but this is mostly possible only with the use of systems which imply irreversible cell damage or destruction. In practice, however, maximum information possible needs to be drawn from noninvasive observations on living spermatozoa, oocytes, and zygotes.

Many abnormalities can be detected by noninvasive examination of zygote pronuclei (also refer to section "Predictive Potential of Zygote Noninvasive Evaluation"). However, pronuclear alterations are nonspecific and can be caused by both sperm-borne factors (50) and by those derived from the oocyte (51). Hence, the identification of the gamete of origin can only be evaluated indirectly, by putting together observations on zygotes and embryos from the present and the past assisted reproduction trials and analyzing them in the overall clinical context of each case.

Sperm-derived, paternal factors are responsible both for abnormalities of fertilization and for defective preimplantation development. The most serious paternal deficiencies can cause complete fertilization failure or a failure of the male pronuclear development. Such conditions can be detected by diagnostic tests using heterologous ICSI with the patient's spermatozoa to mouse, hamster, rabbit, or bovine oocytes (52–54). As mentioned previously, functional abnormalities of the sperm centriole can be revealed by cytochemical visualization of microtubuli in newly formed asters after heterologous ICSI using bovine oocytes (28).

On the other hand, paternal factors often cause much more subtle anomalies. If the two pronuclei fail to get in apposition, the sperm centriole is mostly to be blamed. As to other abnormalities, concerning pronuclear size, orientation, and internal structure, the distinction of the male and the female origin is virtually impossible.

Yet the gamete responsible for fertilization abnormality in question can sometimes be suggested by the clinical context. In an oocyte donation program, for instance, an abnormally high frequency of fertilization abnormalities is likely to be of sperm origin, especially if the infertile couple has experienced a history of similar problems with the patient's own oocytes.

On the other hand, oocyte-derived origin of fertilization abnormalities can be suspected in cases of abnormal hormonal profiles recorded during controlled ovarian stimulation (51). Lack of evidence for a sperm deficiency from a heterologous ICSI test (see above) can further substantiate such a conclusion.

CLINICAL MANAGEMENT OF FERTILIZATION ABNORMALITIES

If a fertilization abnormality is detected, there is hardly anything to be done to rescue the abnormally fertilized oocytes in question. However, this situation should lead to a complex evaluation of the treatment cycle and to a search for alternative with which to cope with. If the problem occurs in the context of conventional IVF, the recourse to ICSI may be considered.

Modification of the ICSI Procedure

There is a body of evidence indicating that complete absence of pronuclear formation after ICSI is mostly caused by a failure of oocyte activation (55,56; reviewed in Ref. 11). Globozoospermia (round-headed sperm syndrome) is a well-defined condition in which the ability of spermatozoa to activate the oocyte is known to be absent or severely impaired (52), although spermatozoa from some globozoospermic patients fertilize normally (57). Moreover, cases of sperm–oocyte-activating factor deficiency, leading to fertilization failure, without the classical globozoospermia phenotype were also observed (57–60). The oocyte, in its turn, may also be responsible for complete fertilization failure, presumably because of deficiencies of the cytoplasmic machinery capable of transducing the sperm-derived signals to adequate effector systems (57).

Both the sperm-borne and the oocyte-borne abnormalities of oocyte activation can now be alleviated in the ICSI setting. Based on initial observations showing that fertilization failure after ICSI can be overcome by subsequent treatment with a calcium mobilizing drug (56,61), several studies reported successful fertilization and ongoing pregnancies in patients with sperm- or oocyte-borne oocyte-activation failures after boosting oocyte activation with a calcium ionophore after ICSI (57–59,62–64). In addition

to the use of ionophores, oocyte activation can also be assisted by other treatments increasing intracellular free calcium concentration in the sperm-injected oocyte, such as electrical stimulation of sperm-injected oocytes (65) or by simple modifications of the ICSI techniques, increasing the intensity or duration of calcium influx from external medium to oocyte cytoplasm during ICSI (57,60).

Unlike complete fertilization failure, alleviation of minor fertilization abnormalities that often lead to the formation of poor-quality embryos is a more complicated issue because of multifactorial etiology of such conditions. If an oocyte factor is suspected, modification of the ovarian stimulation protocol, with continuous readjustment of serum LH concentration (51), may be attempted. In women of >40 years of age ovarian co-stimulation with growth hormone can also be of help (66).

The recourse to more sophisticated gamete selection methods than those currently used in the routine ICSI procedure has also been suggested. These newly developed methods imply visualization of the oocyte meiotic spindle by using a Polscope optical system (67,68) to select the best oocytes for ICSI and the use of high-magnification sperm selection performed with a specially adapted computerized Nomarski interference contrast system working at a total magnification of $\times 6000$ (69,70). Performing ICSI through a laser-drilled opening in the zona pellucida (laser-assisted ICSI) was reported to improve embryo quality in some cases (71), and may also be of help in cases of inherent oocyte fragility. However, the relevance of these new treatment options to cases with minor fertilization abnormalities still remains to be evaluated in large-scale prospective randomized studies.

REFERENCES

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed. New York: Raven Press, 1994:189–317.
2. Jones KT. Ca^{2+} oscillations in the activation of the egg and development of the embryo in mammals. Int J Dev Biol 1998; 42;1–10.
3. Cuthbertson KS, Cobbold PH. Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. Nature 1985; 316:541–542.
4. Taylor CT, Lawrence YM, Kingsland CR, Biljan MM, Cuthbertson KS. Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. Hum Reprod 1993; 8:2174–2179.
5. Tesarik J, Sousa M, Testart J. Human oocyte activation after intracytoplasmic sperm injection. Hum Reprod 1994; 9:511–518.
6. Sousa M, Barros A, Mendoza C, Tesarik J. Effects of protein kinase C activation and inhibition on sperm-, thimerosal-, and ryanodine-induced calcium responses of human oocytes. Mol Hum Reprod 1996; 2:699–708.
7. Tesarik J, Sousa M. Mechanism of calcium oscillations in human oocytes: a two-store model. Mol Hum Reprod 1996; 2:383–386.

8. Tesarik J. Oscillin—reopening the hunting season. Mol Hum Reprod 1998; 4:1007–1009.
9. Tesarik J, Kopecny V. Development of human male pronucleus: ultrastructure and timing. Gamete Res 1989; 24:135–149.
10. Tesarik J, Kopecny V. Nucleic acid synthesis and development of human male pronucleus. J Reprod Fert 1989; 86:549–558.
11. Tesarik J, Mendoza C. In vitro fertilization by intracytoplasmic sperm injection. Bioessays 1999; 21:791–801.
12. Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility. Fertil Steril 1995; 63:1231–1240.
13. Nagy ZP, Liu J, Joris H, et al. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. Hum Reprod 1995; 10:1123–1129.
14. Tesarik J. Using the male gamete for assisted reproduction: past, present and future. J Androl 2003; 24:317–328.
15. Tesarik J. Use of immature germ cells for the treatment of male infertility. Baillière Clin Obstet Gynaecol 1997; 11:763–772.
16. Yanagimachi R. Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. Reprod Biomed Online 2005; 10:247–288.
17. Barak Y, Kogosowski A, Goldman S, Soffer Y, Gonen Y, Tesarik J. Pregnancy and birth after transfer of embryos that developed from single-nucleated zygotes obtained by injection of round spermatids into oocytes. Fertil Steril 1998; 70:67–70.
18. Tesarik J, Sousa M. Comparison of Ca^{2+} responses in human oocytes fertilized by subzonal insemination and by intracytoplasmic sperm injection. Fertil Steril 1994; 62:1197–1204.
19. Sousa M, Mendoza C, Barros A, Tesarik J. Calcium responses of human oocytes after intracytoplasmic injection of leukocytes, spermatocytes and round spermatids. Mol Hum Reprod 1996; 2:853–857.
20. Ozil JP, Swann K. Stimulation of repetitive calcium transients in mouse eggs. J Physiol 1995; 483:331–346.
21. Hewitson LC, Simerly CR, Tengowski MW, et al. Microtubule and chromatin configurations during rhesus intracytoplasmic sperm injection: successes and failures. Biol Reprod 1996; 55:271–280.
22. Bos-Mikich A, Whittingham DG, Jones KT. Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. Dev Biol 1997; 182:172–179.
23. Tesarik J. Oocyte activation after intracytoplasmic injection of mature and immature sperm cells. Hum Reprod 1998; 13(suppl 1):117–127.
24. Tesarik J. Sex chromosome abnormalities after intracytoplasmic sperm injection. Lancet 1995; 346:1096.
25. Asch R, Simerly C, Ord T, Ord VA, Schatten G. The stages at which human fertilization arrests: microtubule and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in humans. Mol Hum Reprod 1995; 1; see Hum Reprod 1995; 10:1897–1906.
26. Van Blerkom J. Sperm centrosome dysfunction: a possible new class of male factor infertility in the human. Mol Hum Reprod 1996; 2:349–354.

27. Obasaju M, Kadam A, Sultan K, Fateh M, Munne S. Sperm quality may adversely affect the chromosome constitution of embryos that result from intracytoplasmic sperm injection. Fertil Steril 1999; 72:1113–1115.
28. Rawe VY, Terada Y, Nakamura S, Chillik CF, Brugo Olmedo S, Chemes HE. A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. Hum Reprod 2002; 17:2344–2349.
29. Tesarik J, Kopecny V. Assembly of the nucleolar precursor bodies in human male pronuclei is correlated with an early RNA synthetic activity. Exp Cell Res 1990; 191:153–156.
30. Tesarik J, Kopecny V. Developmental control of the human male pronucleus by ooplasmic factors. Hum Reprod 1989; 4:962–968.
31. Dvorak M, Tesarik J, Kopecny V. Ultrastructure of human fertilization. In: Van Blerkom J, Motta PM, eds. Ultrastructure of Reproduction. Boston: Martinus Nijhoff, 1984:176–189.
32. Sousa M, Barros A, Silva J, Tesarik J. Developmental changes in calcium content of ultrastructurally distinct subcellular compartments of preimplantation human embryos. Mol Hum Reprod 1997; 3:83–90.
33. Sadowy S, Tomkin G, Munne S. Impaired development of zygotes with uneven pronuclear size. Zygote 1998; 63:137–141.
34. Scott LA, Smith S. The successful use of pronuclear embryo transfers the day following oocyte retrieval. Hum Reprod 1998; 13:1003–1013.
35. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum Reprod 1999; 14:1318–1323.
36. Scott L. Pronuclear scoring as a predictor of embryo development. Reprod Biomed Online 2003; 6:201–214.
37. Salumets S, Hydén-Granskog C, Suikkari AM, Tiitinen A, Tuuri T. The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. Hum Reprod 2001; 16:2177–2181.
38. Tesarik J, Junca AM, Hazout A, et al. Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of pronuclear morphology. Hum Reprod 2000; 15:1396–1399.
39. Scott L, Alvero R, Leondires M, Miller B. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum Reprod 2000; 15:2394–2403.
40. Balaban B, Urman U, Isiklar A, et al. The effect of pronuclear morphology on embryo quality parameters and blastocyst transfer outcome. Hum Reprod 2001; 16:2357–2361.
41. Coskun S, Hellani A, Jaroudi K, Al-Mayman H, Al-Kabra M, Qeba M. Nucleolar precursor body distribution in pronuclei is correlated to chromosomal abnormalities in embryos. Reprod Biomed Online 2003; 7:86–90.
42. Gámiz P, Rubio C, de los Santos MJ, et al. The effect of pronuclear morphology on early development and chromosomal abnormalities in cleavage-stage embryos. Hum Reprod 2003; 18:2413–2419.
43. Balaban B, Yakin K, Urman B, Isiklar A, Tesarik J. Pronuclear morphology predicts embryo development and chromosome constitution. Reprod Biomed Online 2004; 8:695–700.

44. Veeck LL, Amundson CH, Brothman LJ, et al. Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. Fertil Steril 1993; 59:1202–1207.
45. Ludwig M, Schopper B, Al-Hasani S. Clinical use of a pronuclear stage score following intracytoplasmic sperm injection: impact on pregnancy rates under the conditions of the German Embryo Protection Law. Hum Reprod 2000; 15:325–329.
46. Montag M, van der Ven H. Evaluation of pronuclear morphology as the only selection criterion for further embryo culture and transfer: results of a prospective multicenter study. Hum Reprod 2001; 16:2384–2389.
47. Zollner U, Zollner KP, Hartl G, Dietl J, Steck T. The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: the German experience. Hum Reprod 2002; 17:1327–1333.
48. Wittemer C, Bettahar-Lebugle K, Ohl J, Rongières C, Nisand I, Gerlinger P. Zygote evaluation: an efficient tool for embryo selection. Hum Reprod 2000; 15:2591–2597.
49. Rienzi L, Ubaldi F, Iacobelli M, et al. Day 3 embryo transfer with combined evaluation at the pronuclear and cleavage stages compares favourably with day 5 blastocyst transfer. Hum Reprod 2002; 17:1852–1855.
50. Tesarik J, Greco E, Mendoza C. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. Hum Reprod 2000; 17: 184–189.
51. Tesarik J, Mendoza C. Effects of exogenous LH administration during ovarian stimulation of pituitary down-regulated young oocyte donors on oocyte yield and developmental competence. Hum Reprod 2002; 17:3129–3137.
52. Rybouchkin A, Dozortsev D, Pelinck MJ, De Sutter P, Dhont M. Analysis of the oocyte activation capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. Hum Reprod 1996; 11:2170–2175.
53. Nakamura S, Terada Y, Horiuchi T, et al. Analysis of the human sperm centrosomal function and the oocyte activation ability in a case of globozoospermia, by ICSI into bovine oocytes. Hum Reprod 2002; 17:2930–2934.
54. Terada Y, Nakamura S, Morita J, et al. Use of Mammalian eggs for assessment of human sperm function: molecular and cellular analyses of fertilization by intracytoplasmic sperm injection. Am J Reprod Immunol 2004; 51:290–293.
55. Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. Hum Reprod 1994; 9:2374–2380.
56. Tesarik J, Sousa M. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial oocyte activation with calcium ionophore. Fertil Steril 1995; 63:343–349.
57. Tesarik J, Rienzi L, Ubaldi F, Mendoza C, Greco E. Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures. Fertil Steril 2002; 78:619–624.
58. Eldar-Geva T, Brooks B, Margalioth EJ, Zylber-Haran E, Gal M, Silber SJ. Successful pregnancy and delivery after calcium ionophore oocyte activation in a normozoospermic patient with previous repeated failed fertilization after intracytoplasmic sperm injection. Fertil Steril 2003; 79(suppl 3):1656–1658.

59. Chi HJ, Koo JJ, Song SJ, Lee JY, Chang SS. Successful fertilization and pregnancy after intracytoplasmic sperm injection and oocyte activation with calcium ionophore in a normozoospermic patient with extremely low fertilization rates in intracytoplasmic sperm injection cycles. Fertil Steril 2004; 82:475–477.
60. Ebner T, Moser M, Sommergruber M, Jesacher K, Tews G. Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. Hum Reprod 2004; 19:1837–1841.
61. Tesarik J, Testart J. Treatment of sperm-injected human oocytes with Ca^{2+} ionophore supports the development of Ca^{2+} oscillations. Biol Reprod 1994; 51: 385–391.
62. Rybouchkin AV, Van der Straeten F, Quatacker J, De Sutter P, Dhont M. Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil Steril 1997; 68:1144–1147.
63. Kim ST, Cha YB, Park JM, Gye MC. Successful pregnancy and delivery from frozen-thawed embryos after intracytoplasmic sperm injection using round-headed spermatozoa and assisted oocyte activation in a globozoospermic patient with mosaic Down syndrome. Fertil Steril 2001; 75:445–447.
64. Murase Y, Araki Y, Mizuno S, et al. Pregnancy following chemical activation of oocytes in a couple with repeated failure of fertilization using ICSI: case report. Hum Reprod 2004; 19:1604–1607.
65. Yanagida K, Katayose H, Yazawa H, et al. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Hum Reprod 1999; 14:1307–1311.
66. Tesarik J, Hazout A, Mendoza C. Improvement of delivery and live birth rates after ICSI in women aged >40 years by ovarian co-stimulation with growth hormone. Hum Reprod 2005; Advance Access published on April 28, 2005; 20:2536–2541. doi: 10.1093/humrep/dei066.
67. Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. The spindle observation and its relationship with fertilization after intracytoplasmic sperm injection in living human oocytes. Fertil Steril 2001; 75:348–353.
68. Wang WH, Meng L, Hackett RJ, Keefe DL. Developmental ability of human oocytes with or without birefringent spindles imaged by Polscope before insemination. Hum Reprod 2001; 16:1464–1468.
69. Bartoov B, Berkovitz A, Eltes F, et al. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 2003; 80:1413–1419.
70. Berkovitz A, Eltes F, Yaari S, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. Hum Reprod 2004; 20:185–190.
71. Rienzi L, Greco E, Ubaldi F, Iacobelli M, Martinez F, Tesarik J. Laser-assisted intracytoplasmic sperm injection. Fertil Steril 2001; 76:1045–1047.

12

Embryo Development and Assessment of Viability

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Germ cell wastage is a universal phenomenon throughout reproductive life in mammals, including humans. Before puberty and adult life, the vast majority of oocytes become atretic at various stages of follicular development and, of those actually managing to ovulate, only a limited number are capable of repeating the life cycle.

Compared to the natural cycle, the situation in controlled ovarian hyperstimulation is substantially aggravated because accidental maturation and ovulation of germ cells of reduced developmental potential may occur (1). In other words, the actual implantation potential may be overestimated although oocyte morphology, fertilization, and cleavage rate may appear inconspicuous at first glance. On the other hand, even embryos of worst quality may sometimes turn out to be viable, e.g., giving birth to healthy babies.

Taken together, viability of individual embryos is strongly correlated to optimal maturational steps in the ovary, adequate fertilization, progressive development through all pre-implantation stages, as well as subsequent implantation in the endometrium. Combining cytogenetical analysis—morphological evaluation throughout preimplantation development (2), and embryo metabolism (3)—the ability to select the most competent embryo out of a pool of concepti will further improve and definitely help to reach the ultimate goal in assisted reproduction, namely a healthy singleton delivery.

THE FOLLICLE

It is well accepted that the developmental fate of an embryo is largely dictated by the quality of the oocyte, which in turn reflects the follicular milieu.

Most likely, affected gametes are derived from follicles with reduced blood supply since various reports suggest a close relationship between follicular blood flow and developmental competence of the corresponding oocyte or embryo (4,5). If vascularization in ovaries is underdeveloped, some follicles will be confronted with hypoxia which in turn causes a change in energy metabolism by switching from oxidative phosphorylation to glycolysis. As a consequence, adenosine triphosphate (ATP) production in the affected follicle will decrease dramatically, since glycolysis generates only two molecules of ATP compared with oxidative phosphorylation (38 molecules). In addition, ATP depletion is increased since the vast majority of ATP is used for remodeling the vascular network via angiogenesis which is triggered by chronical underoxygenation (6). Since vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis, it can be expected that it is produced by granulosa and theca cells in response to hypoxia. In fact, a significant correlation between elevated levels of VEGF in follicles and a reduced viability of the corresponding embryo has been described (7).

Since conventional parameters, such as follicle size or fluid volume, are not considered to be adequate predictors of developmental potential of harvested oocytes and arising embryos, pulsed color Doppler ultrasound may be the first-line indirect technique for screening for competent oocytes which might serve as a basis for viable embryos or blastocysts, followed by follicular fluid analysis for oxygen, ATP, and/or VEGF.

THE OOCYTE

It is still unknown how follicular underoxygenation affects normal cellular and genetic development of the human oocyte; however, there is evidence that gametes with a reduced internal cytoplasmic pH and ATP content may arise if oxygen saturation falls below a certain threshold of less than or equal to 1% (8).

Nuclear Component

According to Gaulden (9), hypoxia is responsible for a reduction in metabolic activity as well as for a change in internal pH both of which are likely to affect organization and integrity of the meiotic metaphase spindle. This is supported by data from pre-antral follicle culture indicating that in vitro maturation at 5% oxygen tension (instead of 20%) resulted in a significant reduction of gametes finishing nuclear maturation (10), e.g., characterized by a complete spindle absence. More interestingly, the rate of unaligned

chromosomes increased dramatically from 13% in the 20% oxygen group to 35% in the low oxygen cohort. Similarly, Van Blerkom et al. (11) reported that 92% of the oocytes exhibiting chromosome displacement or abnormal chromosomal alignment originated from follicles with dissolved oxygen contents of less than 3%.

Considering the importance of the follicular and in vitro milieu and its close relationship to the health of the gamete, it is not surprising that up to 38% of analyzed MII oocytes lack a spindle apparatus as shown using a polarized light microscope (12). Though detectability improved with increasing PolScope experience (Table 1), one characteristic remained consistent, namely a reduction in developmental competence in spindle negative mature oocytes as assessed by fertilization rate (13,15,16). Even in spindle positive gametes, grading in terms of fertilizability is suggested (16) with those oocytes of worst quality showing a spindle deviation of more than 90 degrees from the first polar body.

However, absence of a birefringent spindle does not predict fertilization failure and developmental arrest. In fact, it has been found that human oocytes with a polar body but without birefringent spindle may still be at telophase I or prometaphase I stage (18). Thus, precocious intracytoplasmic sperm injection (ICSI) in human prometaphase I oocytes with unaligned chromosomes may be one reason why oocytes without a birefringent metaphase II spindle have a significantly worse prognosis.

Knowing the actual position of the birefringent spindle during ICSI can improve embryo quality (14). If no spindle at all has been detected, the probability of a good quality embryo decreases dramatically (13,15), though this suspected correlation is still subject to controversial discussion (16,17).

Table 1 Visualization of Metaphase II Spindle by Means of Polscope and its Influence on Fertilization Rate

Authors	Spindle positive	Spindle in proximity to Pb1	Fertilization rate	
			Spindle	No spindle
Wang et al. (12)	327/533 (61.4)	61 (18.7)	202 (61.8)[a]	91 (44.2)[a]
Wang et al. (13)	1266/1544 (82.0)	Nd	879 (69.4)[b]	175 (62.9)[b]
Cooke et al. (14)	115/124 (92.7)	35 (30.4)	81 (70.4)	Nd
Moon et al. (15)	523/626 (83.6)	252 (48.2)	444 (84.9)[c]	78 (75.7)[c]
Rienzi et al. (16)	484/532 (91.0)	254 (52.5)	362 (74.8)[d]	16 (33.3)[d]
Cohen et al. (17)	585/770 (76.0)	Nd	413 (70.6)[e]	115 (62.2)

[a, b, c, e] $P < 0.05$.
[d] $P < 0.001$.
Abbreviations: Nd: no data available; Pb: first polar body; Values in parentheses are percentages.
Source: From Ref. 18a.

The only paper correlating spindle detection with further preimplantation development to the blastocyst stage (13) reports increased rates of blastocyst development by day five arising from spindle-positive oocytes (51.1%) compared with the spindle-negative counterparts (30.3%), thus supporting the hypothesis that spindle detection may be used as indicator of the oocyte's capacity to form a viable, chromosomally balanced embryo.

In addition, oocytes rather tend to show a visible spindle apparatus if postovulatory age exceeds 38 hours (17), making spindle imaging a new marker for optimal timing of the ICSI procedure and thus increasing the chance to generate viable embryos.

First polar body morphology takes the same line, since the most notable characteristic of postovulatory aging is the spontaneous division or fragmentation of the first polar body (19). Bearing this in mind, it is not surprising that a close correlation between the first polar body appearance and the further fate of the oocyte was observed (20–23). In detail, heavily fragmented first polar bodies were negative predictors of embryo quality, blastocyst formation rate as well as rates of implantation and clinical pregnancy. Apparently this benefit is somewhat reduced with increasing time span between ovulation induction and injection, since a retrospective study applying a different schedule could not find any relationship between constitution of the first polar body and subsequent ICSI outcome (24).

In contrast to postovulatory age, chromosomal status of the oocyte is not reflected by the morphology of the first polar body as suggested from data of polar body biopsy. Regardless of the grade of the first polar body, more than two-thirds of the oocytes were found to be aneuploid (25), but, unfortunately, the most interesting grade consisting of large polar bodies was not analyzed in this highly selected patient cohort.

It has been summarized that MII oocytes of good morphology should be of regular size and show a clear, moderately granulate cytoplasm, a small perivitelline space, and a colorless zona pellucida (2). As a precaution, eggs with an observed deviation in size should not be kept in culture since, e.g., giant oocytes will mostly result in trigynic triploidy (26,27). On the other hand, any reduction in diameter might reflect a certain cytoplasmic loss during manipulation of the oocyte (28).

Cytoplasmic Component

The degree to which cytoplasmic abnormalities, probably being the result of an impaired cytoplasmic maturation, influence fertilizability and further developmental potential is still a matter of debate (29–33). According to Van Blerkom and Henry (34), the further fate of female gametes is dependent on the first occurrence of certain ooplasmic anomalies, e.g., those developing early in maturation may be associated with failed fertilization and aneuploidy while those occurring later in maturation may express developmental

failure despite normal fertilization. However, summarizing the relevant literature dealing with cytoplasmic abnormalities, one may conclude that only few cytoplasmic dysmorphisms actually impair viability of the resultant embryo (29,31,33).

On the one hand, aggregation of the smooth endoplasmic reticulum (sER) was shown to significantly reduce rates of implantation and clinical pregnancy (34), even if transferred embryos did not derive from sER aggregation positive ova, which is presumed to be the result of an underlying adverse factor that might have affected the entire follicular cohort (34). Only one pregnancy went to term after transfer of an embryo developed from an affected gamete (34), and to make matters worse, this baby was diagnosed with Beckwith–Wiedemann syndrome.

On the other hand, vacuolization is the most apparent and dynamic cytoplasmic anomaly in human oocytes. Vacuoles are membrane-bound cytoplasmic inclusions filled with fluid virtually identical with perivitelline fluid and they vary in size as well as in number. It is assumed that vacuoles arise either spontaneously (35) or by fusion of preexisting vesicles derived from the smooth endoplasmic reticulum and/or Golgi apparatus (36).

Recently, a prospective analysis revealed that larger vacuoles above a cut-off value (e.g., 14 μm) affect adequate fertilization and severely impair blastocyst development (37). Two hypotheses could explain these phenomena. First, it is likely that a larger vacuole or multiple vacuoles will cause a much more detrimental effect to the oocyte than a small vacuole since a larger portion of the cytoskeleton (e.g., microtubuli) cannot function as supposed to. Secondly, large vacuoles are thought to displace the MII spindle from its polar position which may result in fertilization failure (35).

Regardless of the different types of cytoplasmic inclusions, it has been observed that a deficiency in ooplasmic texture can also reduce reproductive success. Thus, oocytes with impaired fluidity of the cytoplasm, as assessed by the persistence of the injection funnel after ICSI, had a developmental disadvantage compared to MII gametes with regular viscosity (38). However, extensive cytoplasmic granularity is recognized as the most severe form of cytoplasmic texture anomaly since more than half of affected gametes show chromosomal abnormalities (39), which led to minimal rates of implantation (4.2%) and clinical pregnancy (12.8%).

THE ZYGOTE

Normal fertilization follows a defined course of events, although the timing of these events may vary considerably (for more details, refer to Chapter 11). Either direct deposition (ICSI) or active propulsion [conventional in vitro fertilization (IVF)] ensures presence of a spermatozoon in the cytoplasm. Its head decondenses in the ooplasm prior to the extrusion of the second polar body. The male pronucleus appears in the center of the oocyte

and the female one in close proximity to the meta phase spindle at the periphery of the gamete. Microtubuli growing from the paternal centrosome organize central apposition of both pronuclei (40). This phase is accompanied by final pronuclear growth, nucleolar movement, and coalescence as well as a certain withdrawal of ooplasmic components to the perinuclear region (41).

Abnormal Findings

At least in terms of oocyte polarity, a good quality two-pronuclear zygote is characterized by two polar bodies being located near the pronuclear axis (42). Any deviation from this presumed optimal arrangement that cannot be corrected by microtubuli-driven rotation of the pronuclei (43) could lead to embryos of reduced morphology (42). This drawback is in line with a high rate of complex genetic abnormalities found in embryos derived from zygotes with impaired polarity (44).

However, it may happen that an intrinsic defect of the cytoskeleton or the parental centrosome causes peripheral apposition of both pronuclei (42) or a complete failure in alignment (45), which can result in chromosomal aberrations (44).

The first scenario is more frequent in conventional IVF than in ICSI (3.3 vs. 11.8%), probably due to varying sites of sperm entrance in IVF (42), e.g., spindle-near penetration of the zona could force eccentric formation of pronuclei (46). According to Garello et al. (42), zygotes with eccentric pronuclei show a limited capacity to cleave regularly (47.4%).

The second phenomenon is less frequent in assisted reproduction technologies (approximately 1%) but much more detrimental since the vast majority of zygotes with unaligned pronuclei fail to cleave or show developmental arrest at early stages (47).

Though the female pronucleus usually is smaller than its male counterpart (41), more extensive differences in size (7–10 μm) may occur. This divergence most likely is the result of problems arising during male pronucleus formation (48) and severely affects viability of the corresponding embryos since more than 80% were found to be aneuploid (49,50).

Normal Fertilization

However, the vast majority of zygotes will present with two centrally aligned pronuclei 18 to 20 hours post-insemination. Within these pronuclei, nucleoli tend to align at the pronuclear junction, but since this condition is time-dependent (51), embryologists may be confronted with various pronuclear patterns at the time of fertilization check.

Scott and Smith (45) were the first to report a prognostic value of a zygote score involving pronuclear appearance on implantation and delivery rate. This rather complex score was simplified by focusing exclusively on

pronuclear morphology (47). Thus, it could be shown that interpronuclear synchronicity is a strong predictor of embryo viability (47,52–55). In fact, there is only one report critically questioning this suspected correlation (56), but since it is based on single embryo transfers, it may reflect the actual implantation potential more accurately than studies dealing with double or triple embryo transfers.

In addition to pronuclear pattern, cytoplasmic appearance at zygote stage was part of the original Scott score (45). As demonstrated by time-lapse video cinematography (41), ooplasm withdraws mitochondria and other cell organelles to the perinuclear region during fertilization, leaving a clear halo around the cortex.

Initial studies (56–58) analyzing a suggested relationship between halo formation and outcome (45) were characterized by a lack of standardization in terms of halo scoring, since they either pooled all variations of haloes (e.g., concentric haloes and polar ones) or excluded certain subtypes from analysis. Despite this fact, halo-positivity was found to influence embryo quality (56,59) and blastocyst formation rate (58). Recently, it could be proven that any halo effect, irrespective of its grade and dimension, is of positive predictive power in terms of blastocyst quality and, consequently, clinical pregnancy rate (55).

During evaluation of zygote morphology, it has to be considered that both halo and pronuclear formation follow a fixed schedule. Since direct ooplasmic placement of a viable spermatozoon is performed in ICSI, thus bypassing most steps of fertilization (including acrosome reaction and zona binding), the further course of development will be somewhat accelerated as compared to conventional IVF (60). Consequently, more optimal zygotes were observed in ICSI than in IVF at the time of analysis and pronuclear pattern was performed (54). Therefore, different observation times for microinjected and conventionally inseminated oocytes are recommended.

To summarize, though pronuclear morphology turned out to be an unstable factor within the dynamic process of fertilization, optimal pronuclear patterns, e.g., those with alignment of fused nucleoli, may characterize a subgroup of oocytes showing a developmental advantage compared with zygotes developing more slowly (those showing pronuclear asynchrony).

This is in line with recent findings indicating that during syngamy those zygotes with an accelerated breakdown of the pronuclear membranes (PMB) 22 to 25 hours post-insemination or injection implanted significantly more frequently than those with delayed dissolution (61).

THE CLEAVING EMBRYO

However, just like pronuclear appearance, dissolution of the pronuclei is not a static event and, using it for selection purposes, embryologists may be faced with undocumented zygotes in terms of pronuclear location, size,

and number. Thus, it may happen that, unintentionally, chromosomally imbalanced embryos may be kept in culture if pronuclear morphology could not be checked due to abnormal developmental speed or intense ooplasmic granulation (62,63).

First Cleavage

In this context, first mitotic cleavage (23 to 29 hours after IVF/ICSI) turned out to be a reliable indicator of embryo viability. This morphological criterion is less dynamic than pronuclear patterns, halo formation, or PMB and can be checked easily at first glance. More importantly, it is less time consuming since it does not require additional rotation of the zygotes which sometimes is essential to determine the actual pronuclear pattern. As clearly indicated in Table 2, subdivision of an oocyte pool according to early cleavage behavior seems to be of great benefit in order to assess viability of concepti.

Interestingly, slow cleaving embryos at day 1 were shown to have less blastomeres at later stages of preimplantation development (67), which could be the reason for the observed decrease in blastocyst formation (69), implantation, and clinical pregnancy.

Several reasons may account for this phenomenon. Apart from the fact that, at least in conventional IVF, embryos dividing early may be associated with earlier fertilization, oocyte intrinsic factors are considered to promote early cleavage after fertilization (65). Though currently unknown,

Table 2 Prognostic Relevance of Early Cleavage Behavior on Pregnancy Rate

				Clinical pregnancy rate	
Authors	Method	Hours post IVF/ICSI	Day of transfer	Early cleavage	No cleavage
Shoukir et al. (64)	IVF	25	2	33.3[a]	14.7[a]
Sakkas et al. (65)	ICSI	27	2	25.9[b]	3.2[b]
Sakkas et al. (66)	IVF/ICSI	23–27	2	45.0[c]	23.8[c]
Lundin et al. (67)	IVF/ICSI	25–27	2, 3	40.5[d]	31,3[d]
Bos-Mikich et al. (68)	IVF/ICSI	25–29	3	54.8[e]	25.0[e]
Fenwick et al. (69)	IVF	25	2	31.3[f]	10.5[f]
Salumets et al. (70)	IVF/ICSI	25–27	2	50.0[g]	26.4[g]
Windt et al. (71)	ICSI	26	2, 3	37.5[h]	11.1[h]
Van Montfoort et al. (72)	IVF/ICSI	23–28	2	37.1[i]	10.3[i]

[a, b, e, f] $P < 0.05$.
[d, g] $P < 0.01$.
[c, h, i] $P < 0.001$.
Abbreviations: IVF, in-vitro fertilization; ICSI, intracytoplasmic sperm injection.
Soruce: From Ref. 72a.

such factors could be related to the expression of human leukocyte antigen G (73), a candidate human functional homolog to the mouse Qa-2 antigen, which, as a product of the preimplantation embryo development (Ped) gene, promotes rapid mitotic divisions. An alternative explanation would be that slow cleaving human embryos have an early lag phase in cell cycle, which was found to be detrimental to blastocyst rate of bovine embryos (74).

Whether a genetic predisposition influences viability of the developing embryo or to what extent metabolic disturbances (e.g., mitochondrial content, ATP production, mRNA, cytoplasmic maturation) cause late cleavage is still matter of discussion. However, several data from embryo culture rather support a genetic reason. On the one hand, it could be documented that early dividing embryos show a lower rate of multinucleated blastomeres (70), and on the other hand, tripronuclear zygotes show a limited capacity to cleave early as compared to their binucleated counterparts (67).

Further Cleavages

As cleavage continues, the first blastomeres of the two-cell embryo divides meridionally followed by approximately equatorial cleavage of the other cell (75). This lesson from mouse embryos may explain the typical crosswise appearance of the 4-cell human conceptus on day two of development. Although a regular tetrahedral configuration of blastomeres with six intercellular contacts is the most common outcome of second cleavage, both the distribution and the relationship between blastomeres may vary, including specimens that are essentially planar. This arrangement involves a reduced number of cell–cell contacts which could impair compaction and delay blastulation of the embryo (28,76).

Apart from the number of blastomeres, routine assessment of embryo quality from day two onward also includes the degree of fragmentation. There is a considerable lack of objective and standardized methods for assessing embryonic fragmentation. In fact, a cell size of 45 μm on day two has been suggested which allowed distinguishing between anuclear fragments and blastomeres. Below this cut-off value, only 3% of the cells contained DNA compared to 67% with a diameter above this cut-off. Similar results were published for day three embryos with the exception that, due to ongoing cleavage, a threshold of 40 μm was indicative in terms of differentiation (77).

It is generally accepted that minor fragmentation does not impair viability of the embryo (78,79) and may disappear during in vitro culture, either by lysis or resorption (80,81). Larger amounts of fragments, however, significantly reduce the chance to achieve pregnancy (82) and, even more importantly, perinatal outcome of babies derived from heavily fragmented embryos (greater than equal to 50% fragmentation) was found to be worse compared with that after transfer of more or less fragment-free embryos (83).

As fragments are structures of blastomeric origin, the actual amount of cytoplasmic fragmentation during cleavage stage can be estimated by the difference between the previous zygote volume and the overall blastomere volume (84). In cases of moderate fragmentation, it appears that different spatial patterns of fragmentation are of more severe developmental consequences than fragmentation per se (81,82). In detail, smaller, more localized fragments did not impair viability, whereas larger and more scattered fragments had a disastrous effect on implantation (82). Theoretically, the detrimental effect of such patterns may be explained by the fact that anuclear fragments lying in close proximity to an assumed cleavage axis may impair further cleavage and/or reduce the number of cell-to-cell contacts required for regular compaction and blastocyst formation. Viability of bad quality embryos may be improved in certain instances if spatial relationship of blastomeres is restored by cosmetical removal of the acellular remnants (82).

The higher the degree of blastomeric decay, the higher the risk of chromosomal imbalances, such as mosaicism (63). In these cases, selective fragmentation could function as a means to completely exclude affected blastomeres from further cell aggregation (85). This process is most likely related to programmed cell death (85,86), e.g., the ratio of the two apoptosis-related gene families bcl-2 and bax (85). Others (87) question a direct relationship between apoptotic phenomena and fragmentation and much rather speculate that apoptosis may be triggered if the degree of mitochondrial and proteinic loss due to fragmentation reaches a certain level.

It is an undisputed fact that two morphological phenomena, namely multinucleation and inequality of cleavage, reduce viability of the cleaving embryos to a minimum. Frequently, both anomalies coincide (80), which may be explained by the larger cell size of multinucleated blastomeres (84). In general, it can be expected that about one-third of all day two and three embryos show at least one multinucleated blastomere (88). The overall incidence, however, will generally be underestimated since nuclei are only visible at interphase. Most previous studies report a disastrous implantation rate of less than 6% after exclusive transfer of bi-or multinucleated embryos (88,89). This reduced outcome seems to be a reflection of the chromosomal constitution of the embryos since the vast majority of them (approximately 75%) were chromosomally abnormal (80,90). In detail, cytokinesis may fail during any mitotic division (91) with the worst outcome to be expected if problems arise during the first cleavage ending up with both cells being multinucleated (92).

Even if the embryo is composed of a stage-appropriate number of equally divided mononuclear cells, this does not mean that the texture of the blastomeres will correspond to the assumed normal condition, which is a translucent cytoplasm with moderate granulation. As a result of culture conditions (e.g., media composition), cytoplasm of a day 3 embryo may change to a more mottled appearance showing numerous small (1.5 μm) pits

on the surface (93,94). Pitting is physically different from excessive granulation and mostly affects embryos after embryonic genome activation (93). In humans, this switch from maternal genome control is considered to take place around the 8-cell stage (95). Definitely, it is an important hallmark of preimplantation development prior to which it is an assessment of oocyte quality rather than embryo viability.

However, this temporal coincidence of cytoplasmic pitting is not a positive predictor of outcome (96); but much rather it seems to be completely unrelated to implantation and pregnancy. Recent findings, however, suggest a certain influence on viability since some 30% of implantations vanished after exclusive transfer of pitted embryos compared to only 16% of early pregnancy loss in a nonaffected control group (97).

Compacting

While at earlier cleavage stages embryos resemble an accumulation of solitary blastomeres with a rudimentary level of biosynthesis, compaction phase (beginning on day 3) is characterized by increased biosynthetic rates and the capacity to metabolize glucose more efficiently. In addition, the compacting embryo is capable of actively regulating ionic gradients, thus controlling its internal environment (98).

Compaction is due to the formation and the number of tight intercellular junctions (e.g., desmosomes, gap, and tight junctions) causing blastomeres to become closely apposed (76,99). Due to this highly interactive cell mass, blastomeres loose their totipotent characteristics.

In humans, compaction begins around eight-cell stage probably following an intrinsic developmental clock. Precocious compacting at day 2 could result in formation of trophoblastic vesicles leaving no predecessor cells of inner cell mass (6). On the other hand, 16-cell embryos without the slightest evidence of compaction are of reduced capacity and will hardly reach blastocyst stage (99).

Tao and co-workers (100) successfully tried to predict implantation scoring embryos at the compaction stage. These authors showed that the implantation potential is positively related to the proportion of blastomeres undergoing compaction. Consequently, embryos had the worst prognosis if less than half of the blastomeres were involved in the compaction process. Blastomeres and fragments that are unable to form appropriate contacts are generally excluded from the compaction process and remain within the empty zona pellucida after hatching (99).

EMBRYO METABOLISM

Despite the fact that numerous morphological criteria have been published which could add to predictive power on further developmental potential of

day 3 embryos, there is a tendency to question a close correlation between overall day 3 morphology and blastocyst formation as well as quality (101–103).

In this regard, biochemical criteria could be more appropriate to filter out those embryos with metabolic activity within normal range, and thus identify those embryos which will preferentially proceed to blastocyst stage. However, the required techniques (e.g., ultramicrofluorescence, high-performance liquid chromatography) usually are not available in standard IVF laboratories, severely limiting their application in routine work.

Depending on developmental stage, metabolic activity and profile of the embryos may differ enormously. Though sequential culture media try to imitate uterine milieu (for more details, refer to Chapter 13) and therefore fulfill all major requirements of the growing embryo, not all of the concepti can adapt to the different environment. This incompetence may be expressed as a change in metabolic pattern which in turn may suggest reduced viability to the embryologist.

Both glucose uptake and lactate production proved useful in quantifying glycolytic activity, which was then used to prospectively select mouse blastocysts for transfer (3). Out of a pool of blastocysts of similar expansion and morphology, those blastocysts with glycolytic activity closest to that observed for blastocysts developed in vivo showed the highest fetal development rate (80%). An abnormal rate of glycolysis as expressed as excessive lactate production led to a decrease in fetal development (6%).

The authors (104) also utilized carbohydrate metabolism as a means to predict blastocyst formation in human embryos. Pyruvate, as well as glucose uptake, were significantly higher in embryos that went to blastocyst stage than in embryos with developmental arrest. Much more interestingly, glucose (but not pyruvate) uptake was highest in blastocysts of highest grade, emphasizing the importance of glucose uptake in terms of noninvasive selection.

A similar approach is the noninvasive analysis of amino acid turnover. It could be demonstrated that day 2 or 3 embryos with a future competence to form blastocysts exhibit amino acid flux patterns distinct from those in embryos with comparable morphology that stop development (105). In those embryos that progressed to blastocyst stage, leucin was the only amino acid being significantly depleted. This fact may emphasize the presumed role of this essential amino acid as a stimulator of protein synthesis. Alanine was the most striking amino acid found to have a net appearance, probably due to its involvement in disposal of embryotoxic ammonium ions (105). Nonviable embryos showed a 3.7-fold greater amino acid turnover than competent concepti, strongly indicating a degeneration of metabolism similar to the negative effect of rather excessive glycolytic activity (3). Further studies suggested that amino acids whose turnover predicted blastulation are different from those predicting pregnancy and life birth (106). This may reflect the fact that not all blastocysts forming in vitro are as viable as they are expected based on their morphological appearance.

THE BLASTOCYST

After compaction of the cleaving embryo, it begins to form a cavity. During blastocyst formation, two clearly distinguishable cell lines are formed, namely the trophectoderm and the inner cell mass. In humans, the proliferation of the latter was found to be 1.5 times lower than that of the trophectoderm (107). Similar to the developmental stage of the blastocyst on days 5 and 6, which may range from a retarded morula to an expanded or hatching blastocyst, a high variability in cell number has been observed. A full human blastocyst at day 5 of development should exceed 60 cells and should at least have doubled its cell number on day 6 (107,108). It should be noted that blastocyst formation in vitro is not a reliable marker of chromosomal balance since certain genetic aberrations are compatible with good morphology at blastocyst stage (109,110).

Expansion of the blastocyst and appearance of both cell lineages were taken into account for scoring blastocyst morphology (111) and were successfully used in an IVF programme (112,113). According to this grading system (111), viable blastocysts are characterized by a cohesive trophectoderm composed of numerous sickle-shaped cells as well as a tightly packed inner cell mass. In detail, such top quality blastocysts showed implantation and pregnancy rates as high as 70 and 87%, respectively, in double blastocyst transfers and 50 and 70% in single transfers (112).

It turned out that the expansion of the blastocyst on day 5 is the less predictive parameter in terms of implantation (114,115). Nevertheless, other investigators (116) reported highest viability in day 5 blastocysts, which were expanded and derived from day 3 embryos of adequate blastomere number (7–8 cells). This slight divergence may be explained by the simple fact that quality of inner cell mass can only be evaluated from full blastocyst stage onward (112), whereas morula and early blastocyst stage does not allow for detailed inner cell mass scoring. The size and shape of this cell lineage, however, was highly predictable in terms of implantation and pregnancy (114). In detail, slightly oval inner cell masses above 4500 μm^2 were of highest viability. On the other hand, necrotic foci within the inner cell mass were correlated with a decrease in subsequent viability (115). Such bad quality blastocysts usually show lower cell numbers and a higher degree of chromosomal aberrations (117).

Cells constituting the trophectoderm may also display abnormal features, e.g., a deviation in number or shape; however, due to an increased cleavage rate (107), the actual impact of trophectoderm morphology on viability is somewhat limited. During blastocyst expansion, trophectodermal cells situated in close proximity to the inner cell mass migrate from this region to populate the mural end. During this migration, the cells stay attached to the inner cell mass via cytoplasmic strings that withdraw as they reach their final location (118). Persistence of these cytoplasmic strings until

expanded blastocyst stage substantially impairs embryo quality and polarity and is associated with a reduced outcome (119).

CONCLUSION

Considering the fact that certain potential methods of predicting embryo viability, e.g., adequate measurement of follicles vascularization or correct

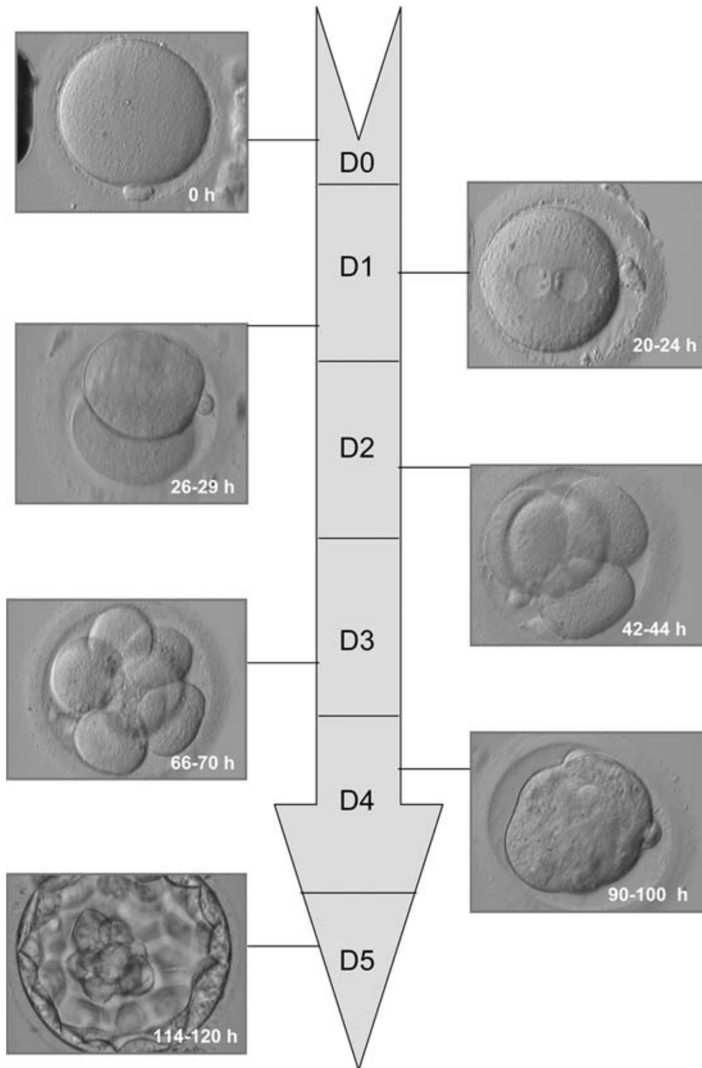

Figure 1 Timeline for optimal blastocyst development.

assessment of embryo metabolism, are hardly applicable in routine IVF laboratories, embryologists have to rely on the limited prognostic power of morphological criteria at different stages of preimplantation development (2,6). In fact, sequential assessment at different developmental stages allowed for an increased prediction of blastocyst formation (120) and pregnancy (121–123).

Strategies to optimize selection based on morphological criteria should include elimination of gametes or concepti with suspected chromosomal imbalance (2). Thus, giant oocytes should never be inseminated or injected (26,27). In addition, oocytes showing dense central granulation (39) or aggregation of smooth endoplasmic reticulum (34) should be separated. At cleavage stages, multinucleation and the presence of unequal blastomeres (79,80) as well as large amounts of fragmentation (83) should be considered as potential sources of aneuploidy.

Once these candidates with poor prognosis have been eliminated from the pool of embryos/blastocysts considered for transfer those concepti should be chosen which follow a specific time line of preimplantation development (Fig. 1) but also combine as many positive predictors as possible.

By doing so, routine single embryo transfer will become a feasible objective. This would definitely help to reduce multiple pregnancy and malformation rates, the ultimate goals of assisted reproductive technologies.

REFERENCES

1. Serhal PF, Ranieri DM, Kinis A, et al. Oocyte morphology predicts outcome of intracytoplasmic sperm injection. Hum Reprod 1997; 12:1267–1270.
2. Ebner T, Moser M, Sommergruber M, et al. Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development. Hum Reprod Update 2003; 9:251–262.
3. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod 1996; 11:1975–1978.
4. Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum Reprod 1997; 12:1047–1055.
5. Huey S, Abuhamad A, Barroso G, et al. Perifollicular blood flow Doppler indices but not follicular pO_2, pCO_2, or pH, predict oocyte developmental competence in in vitro fertilization. Fertil Steril 1999; 72:707–712.
6. Scott L. The biological basis of non-invasive strategies for selection of human oocytes and embryos. Hum Reprod Update 2003; 9:237–249.
7. Barroso G, Barrionuevo M, Rao P, et al. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril 1999; 72:1024–1026.
8. Van Blerkom J. The influence of intrinsic an extrinsic factors on the developmental potential of chromosomal normality of the human oocyte. J Soc Gyn Invest 1996; 3:3–11.

9. Gaulden M. Maternal age effect: the enigma of down syndrome and other trisomic conditions. Mutat Res 1992; 296:69–88.
10. Hu Y, Betzendahl I Cortvrindt R, et al. Effects of low O_2 and aging on spindles and chromosomes in mouse oocytes from pre-antral follicle culture. Hum Reprod 2001; 16:737–748.
11. Wang WH, Meng L, Hackett RJ, et al. The spindle observation and its relationship with fertilization after intracytoplasmic sperm injection in living human oocytes. Fertil Steril 2001; 75:348–353.
12. Wang WH, Meng L, Hackett RJ, et al. Developmental ability of human oocytes with or without birefringent spindles imaged by polyscope before insemination. Hum Reprod 2001; 16:1464–1468.
13. Moon JH, Hyum CS, Lee SW, et al. Visualization of the metaphase II meiotic spindle in living human oocytes using the polyscope enables the prediction of embryonic developmental competence after ICSI. Hum Reprod 2003; 18:817–820.
14. Rienzi L, Ubaldi F, Martinez F, et al. Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI. Hum Reprod 2003; 18:1289–1293.
15. Eichenlaub-Ritter U, Shen Y, Tinneberg HR. Manipulation of the oocyte: possible damage to the spindle apparatus. Reprod Biomed Online 2002; 5:117–124.
16. Cooke S, Tyler JP, Driscoll GL. Meiotic spindle location and identification and its effect on embryonic cleavage plane and early development. Hum Reprod 2003; 18:2397–2405.
17. Cohen Y, Malcov M, Schwartz T, et al. Spindle imaging: a new marker for optimal timing of ICSI. Hum Reprod 2004; 19:649–654.
18. Eichenlaub-Ritter U, et al. Manipulation of the oocyte: possible damage to the spindle apparatus. Reprod Biomed Online 2002; 5:117.
18a. Ebner T, Moser M, Sommer gruber M, et al. Die Bedeutung der Analyse der Metaphase-II-Spindel in der assistierten Reproduktion. J Reproduktions med Endokrinol 2005; 2:78.
19. Choi T, Fukasawa K, Zhou R, et al. The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proc Natl Acad Sci USA 1996; 93:7032–7035.
20. Xia P. Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. Hum Reprod 1997; 12:1750–1755.
21. Ebner T, Moser M, Yaman C, et al. Elective transfer of embryos selected on the basis of first polar body morphology is associated with increased rates of implantation and pregnancy. Fertil Steril 1999; 72:599–603.
22. Ebner T, Yaman C, Moser M, et al. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. Hum Reprod 2000; 15:427–430.
23. Ebner T, Moser M, Sommergruber M, et al. First polar body morphology and blastocyst formation rate in ICSI patients. Hum Reprod 2002; 17:2415–2418.
24. Ciotti PM, Notarangelo L, Morselli-Labate AM, et al. First polar body morphology before ICSI is not related to embryo quality or pregnancy rate. Hum Reprod 2004; 19:2334–2339.

25. Verlinsky Y, Lerner S, Illkevitch N, et al. Is there any predictive value of first polar body morphology for embryo genotype or developmental potential. Reprod Biomed Online 2003; 7:336–341.
26. Balakier H, Bouman D, Sojecki A, et al. Morphological and cytogenetic analysis of human giant oocytes and giant embryos. Hum Reprod 2002; 17: 2394–2401.
27. Rosenbusch B, Schneider M, Glaser B, et al. Cytogenetic analysis of giant oocytes and zygotes to assess their relevance for the development of digynic triploidy. Hum Reprod 2002; 17:2388–2393.
28. Ebner T, Moser M, Tews G. Intracytoplasmic sperm injection in zona-free oocytes. J Turkish German Gynecol Assoc 2004; 5:294–298.
29. Alikani M, Palermo G, Adler A, et al. Intracytoplasmic sperm injection in dysmorphic human oocytes. Zygote 1995; 3:283–288.
30. De Sutter P, Dozortsev D, Qian C, et al. Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. Hum Reprod 1996; 11:595–597.
31. Balaban B, Urman B, Sertac A, et al. Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection. Hum Reprod 1998; 13:3431–3433.
32. Loutradis D, Drakakis B, Kallianidis K, et al. Oocyte morphology correlates with embryo quality and pregnancy rate after intracytoplasmic sperm injection. Fertil Steril 1999; 72:240–244.
33. Van Blerkom J, Henry G. Oocyte dysmorphism and aneuploidy in meiotically mature oocytes after ovarian stimulation. Hum Reprod 1992; 7:379–390.
34. Otsuki J, Okada A, Morimoto K, et al. The relationship between pregnancy outcome and smooth endoplasmic reticulum clusters in MII human oocytes. Hum Reprod 2004; 19:1591–1597.
35. Van Blerkom J. Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. J Electron Microsc Tech 1990; 16:324–346.
36. El.Shafie M, Windt ML, Kitshoff M, et al. Ultrastructure of human oocytes: a transmission electron microscopic view. In: El.Shafie M, Sousa M, Windt ML, et al., eds. An Atlas of the Ultrastructure of Human Oocytes. New York: Parthenon Publishing, 2000.
37. Ebner T, Moser M, Sommergruber M, et al. Occurrence and developmental consequences of vacuoles throughout preimplantation development. Fertil Steril 2005; 83:1635–1640.
38. Ebner T, Moser M, Sommergruber M, et al. Developmental competence of oocytes showing increased cytoplasmic viscosity. Hum Reprod 2003; 18:1294–1298.
39. Kahraman S, Yakin K, Donmez E, et al. Relationship between granular cytoplasm of oocytes and pregnancy outcome following intracytoplasmic sperm injection. Hum Reprod 2000; 15:2390–2393.
40. Schatten G. The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its resaturation during fertilization. Dev Biol 1994; 165:299–335.

41. Payne D, Flaherty SP, Barry MF, et al. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. Hum Reprod 1997; 12:532–541.
42. Garello C, Baker H, Rai J, et al. Pronuclear orientation, polar body placement, and embryo quality after intracytoplasmic sperm injection and in-vitro fertilization: further evidence for polarity in human oocytes. Hum Reprod 1999; 14:2588–2595.
43. Edwards RG, Beard H. Oocyte polarity and cell determination in early mammalian embryos. Mol Hum Reprod 1997; 3:863–905.
44. Gianaroli L, Magli MC, Ferraretti AP, et al. Pronuclear morphology and chromosomal abnormalities as scoring criteria for embryo selection. Fertil Steril 2003; 80:341–349.
45. Scott LA, Smith S. The successful use of pronuclear embryo transfers the day following oocyte retrieval. Hum Reprod 1998; 13:1003–1013.
46. Van Blekom J, Davis P, Merriam J, et al. Nuclear and cytoplasmic dynamics of sperm penetration, pronuclear formation, and microtubule organization during fertilization and early preimplantation development in the human. Hum Reprod Update 1995; 1:429–461.
47. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum. Reprod 1999; 14:1318–1323.
48. Goud P, Goud A, Diamond MP, et al. Fertilization abnormalities und pronucleus size asynchrony after intracytoplasmic sperm injection is related to oocyte post maturity. Fertil Steril 1999; 72:245–252.
49. Sadowy S, Tomkin G, Munne S, et al. Impaired development of zygotes with uneven pronuclear size. Zygote 1998; 6:137–141.
50. Manor D, Drugan A, Stein D, et al. Unequal pronuclear size—a powerful predictor of embryonic chromosome anomalies. J Assist Reprod Genetics 1999; 16:385–389.
51. Wright G, Wiker S, Elsner C, et al. Observations on the morphology of pronuclei und nucleoli in human zygotes und implications for cryopreservation. Hum Reprod 1990; 5:109–115.
52. Wittemer C, Bettahar-Lebugle K, Ohl J, et al. Zygote evaluation: an efficient tool for embryo selection. Hum Reprod 2000; 15:2591–2597.
53. Balaban B, Urman B, Isiklar A, et al. The effect of pronuclear morphology on embryo quality parameters und blastocyst transfer outcome. Hum Reprod 2001; 16:2357–2361.
54. Montag M, van der Ven H. Evaluation of pronuclear morphology as the only selection criterion for further embryo culture und transfer: results of a prospective multicentre study. Hum Reprod 2001; 16:2384–2389.
55. Ebner T, Moser M, Sommergruber M, et al. Presence, but not type or degree of extension, of a cytoplasmic halo has a significant influence on preimplantation development and implantation behaviour. Hum Reprod 2003; 18:2406–2412.
56. Salumets A, Hyden-Granskog C, Suikkari AM, et al. The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. Hum Reprod 2001; 16:2177–2181.

57. Stalf T, Herrero J, Mehnert C, et al. Influence of polarization effects und pronuclei on embryo quality und implantation in an IVF program. J Assist Reprod Genet 2002; 19:355–362.
58. Zollner U, Zollner KP, Hartl G, et al. The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: the German experience. Hum Reprod 2002; 17:1327–1333.
59. Scott LA. Pronuclear scoring as a predictor of embryo development. Reprod Biomed Online 2003; 6:201–214.
60. Nagy ZP, Janssenswillen C, Janssens R, et al. Timing of oocyte activation, pronucleus formation, and cleavage in humans after intracytoplasmic sperm injection (ICSI) with testicular spermatozoa and after ICSI or in-vitro fertilization on sibling oocytes with ejaculated spermatozoa. Hum Reprod 1998; 13:1606–1612.
61. Fancsovits P, Toth L, Murber A, et al. Early pronuclear breakdown is a good indicator of embryo quality and viability. Fertil Steril 2005; 84:881–887.
62. Manor D, Kol S, Lewit N, et al. Undocumented embryos: do not trash them FISH them. Hum Reprod 1996; 11:2502–2506.
63. Munné S, Cohen J. Chromosome abnormalities in human embryos. Hum Reprod Update 1998; 4:842–855.
64. Shoukir Y, Campana A, Farley T, et al. Early cleavage of in-vitro fertilized human embryos to the 2–cell stage: a novel indicator of embryo quality und viability. Hum Reprod 1997; 12:1531–1536.
65. Sakkas D, Shoukir Y, Chardonnens D, et al. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum Reprod 1998; 13:182–187.
66. Sakkas D, Percival G, D'Arcy Y, et al. Assessment of early cleaving in in vitro fertilized human embryos at the 2-cell stage before transfer improves embryo selection. Fertil Steril 2001; 76:1150–1156.
67. Lundin K, Bergh C, Hardarson T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. Hum Reprod 2001; 16:2652–2657.
68. Bos-Mikich A, Mattos AL, Ferrari AN. Early cleavage of human embryos: an effective method for predicting successful IVF/ICSI outcome. Hum Reprod 2001; 16:2658–2661.
69. Fenwick J, Platteau P, Murdoch AP, et al. Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. Hum Reprod 2002; 17:407–412.
70. Salumets A, Hyden-Granskog C, Makinen S, et al. Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. Hum Reprod 2003; 18:821–825.
71. Windt ML, Kruger TF, Coetzee K, et al. Comparative analysis of pregnancy rates after the transfer of early dividing embryos versus slower dividing embryos. Hum Reprod 2004; 19:1155–1162.
72. Van Montfoort APA, Dumoulin JC, Kester AD, et al. Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers, Hum Reprod 2004; 19:2103–2108.
72a. Ebner T, Moser M, Sommergruber M, et al. Neue Aspekte der Zygoten evaluierung. J Reproduktions med Endokrinol 2004; 2:71.

73. Jurisicova A, Casper RF, MacLusky NJ, et al. HLA-G expression during preimplantation human embryo development. Proc Natl Acad Sci 1996; 93: 161–165.
74. Grisart B, Massip A, Dessy F. Cinematographic analysis of bovine embryo development in serum-free oviduct-conditioned medium. J Reprod Fertil 1994; 101:257–264.
75. Gardner RL. Experimental analysis of second cleavage in the mouse. Hum Reprod 2002; 17:3178–3189.
76. Suzuki H, Togashi M, Adachi J, et al. Developmental ability of zona-free mouse embryos is influences by cell association at the 4-cell stage. Biol Reprod 1995; 53:78–83.
77. Johansson M, Hardarson T, Lundin K. There is a cut-off limit in diameter between a blastomere and a small anucleate fragment. J Assist Reprod Genet 2003; 20:309–313.
78. Ziebe S, Petersen K, Lindenberg S, et al. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Hum Reprod 1997; 12:1545–1549.
79. Van Royen E, Mangelschots K, De Neubourg D, et al. Characterization of a top quality embryo, a step towards single embryo transfer. Hum Reprod 1999; 14:2345–2349.
80. Hardarson T, Hanson C, Sjogren A, et al. Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. Hum Reprod 2001; 16:313–318.
81. Van Blerkom J, Davis P, Alexander S. A microscopic and biochemical study of fragmentation phenotypes in stage appropriate human embryos. Hum Reprod 2001; 16:719–729.
82. Alikani M, Cohen J, Tomkin G, et al. Human embryo fragmentation in vitro and its implications for pregnancy and implantation. Fertil Steril 1999; 71:836–842.
83. Ebner T, Yaman C, Moser M, et al. Embryo fragmentation in vitro and its impact on treatment and pregnancy outcome. Fertil Steril 2001; 76:281–285.
84. Hnida C, Engenheiro E, Ziebe S. Computer-controlled, multilevel, morphometric analysis of blastomere size as biomarker of fragmentation and multinuclearity in human embryos. Hum Reprod 2004; 19:288–293.
85. Warner CM, Cao W, Exley GE, et al. Genetic regulations of egg and embryo survival. Hum Reprod 1998; 13(suppl 3):178–190.
86. Jurisicova A, Varmuza S, Casper RF. Programmed cell death and human embryo fragmentation. Mol Hum Reprod 1996; 2:93–98.
87. Antczak M, Van Blerkom J. Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. Hum Reprod 1999; 14:429–447.
88. Van Royen E, Mangelschots K, Vercruyssen M, et al. Multinucleation in cleavage stage embryos. Hum Reprod 2003; 18:1062–1069.
89. Pelinck MJ, De Vos M, Dekens M, et al. Embryos cultured in vitro with9multinucleated blastomeres have poor implantation potential in human in-vitro fertilization and intracytoplasmic sperm injection. Hum Reprod 1998; 13:960–963.

90. Kligman I, Benadiva C, Alikani M, et al. The presence of multinucleated blastomeres in human embryos correlates with chromosomal abnormalities. Hum Reprod 1996; 11:1492–1498.
91. Hardy K, Winston RM, Handyside AH. Binucleate blastomeres in preimplantation human embryos in vitro: failure of cytokinesis during early cleavage. J Reprod Fertil 1993; 98:549–558.
92. Balakier H, Cadesky K. The frequency and developmental capability of human embryos containing multinucleated blastomeres. Hum Reprod 1997; 12: 800–804.
93. Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in $KSOM^{AA}$ medium: is a two-step protocol necessary. Reprod BioMed Online 2002; 5:133–140.
94. Cooke S, Quinn P, Kime L, et al. Improvement in early human embryo development using new formulation sequential stage-specific culture media. Fertil Steril 2002; 78:1254–1260.
95. Braude PR, Bolton VN, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 1988; 332:459–461.
96. Rienzi L, Ubaldi F, Minasi MG, et al. Blastomere cytoplasmic granularity is unrelated to developmental potential of day 3 human embryos. J Assist Reprod Genet 2003; 20:314–317.
97. Ebner T, Tews G, Sommergruber M, et al. Cytoplasmic pitting has a negative impact on implantation outcome. J Assist Reprod Genet 2005; 22:239–244.
98. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF. Hum Reprod Update 1997; 3:367–382.
99. Veeck LL, Zaninovic N. Human morulae in vitro. In: Veeck LL, Zaninovic N, eds. An Atlas of Human Blastocysts. Boca Raton: Parthenon Publishing, 2003.
100. Tao J, Tamis R, Fink K, et al. The neglected morula/compact stage embryo transfer. Hum Reprod 2002; 17:1513–1518.
101. Rijnders PM, Jansen CAM. The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. Hum Reprod 1998; 13:2869–2873.
102. Graham J, Han T, Porter R, et al. Day 3 morphology is a poor predictor of blastocyst quality in extended culture. Fertil Steril 2000; 74:495–497.
103. Milki AA, Hinckley MD, Genhardt J, et al. Accuracy of day 3 criteria for selecting the best embryo. Fertil Steril 2002; 77:1191–1195.
104. Gardner DK, Lane M, Stevens J, et al. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril 2001; 76:1175–1180.
105. Houghton FD, Hawkhead JA, Humpherson PG, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod 2002; 17:999–1005.
106. Brison DR, Houghton FD, Falconer D, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod 2004; 19:2319–2324.

107. Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development 1989; 107:597–604.
108. Fong CY, Bongso A. Comparison of human blastulation rates and total cell number in sequential culture media with and without co-colture. Hum Reprod 1999; 14:774–781.
109. Magli MC, Jones MG, Gras L, et al. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Hum Reprod 2000; 15:1781–1786.
110. Sandalinas M, Sadowy S, Alikani M, et al. Developmental ability of chromosomal abnormal human embryos to develop to the blastocyst stage. Hum Reprod 2001; 16:1954–1958.
111. Gardner DK, Schoolcraft WB. In vitro culture of human blastocyst. In: Jansen R, Mortimer D, eds. Towards Reproductive Certainty: Infertility and Genetics Beyond 1999. Carnforth: Parthenon Press, :378.
112. Gardner DK, Lane M, Stevens J, et al. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 2000; 73:1155–1158.
113. Balaban B, Urman B, Sertac A, et al. Blastocyst quality affects the success of blastocyst-stage embryo transfer. Fertil Steril 2000; 74:282–287.
114. Richter KS, Harris DC, Daneshmand ST, et al. Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. Fertil Steril 2001; 76:1157–1167.
115. Kovacic B, Vlaisavljevic V, Reljic M, et al. Developmental capacity of different morphological types of day 5 human morulae and blastocysts. Reprod Biomed Online 2004; 8:687–694.
116. Racowsky C, Combelles CM, Nureddin A, et al. Day 3 and day 5 morphological predictors of embryo viability. Reprod Biomed Online 2003; 6:323–331.
117. Hardarson T, Caisander G, Sjogren A, et al. A morphological and chromosomal study of blastocysts developing from morphologically suboptimal human pre-embryos compared with control blastocysts. Hum Reprod 2003; 18:399–407.
118. Gardner R. Flow of cells from polar to mural trophectoderm is polarized in the mouse blastocyst. Hum Reprod 2000; 15:694–701.
119. Scott L. Oocyte and embryo polarity. Sem Reprod Med 2000; 18:171–183.
120. Neuber E, Rinaudo P, Trimarchi JR, et al. Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development. Hum Reprod 2003; 18:1307–1312.
121. De Placido G, Wilding M, Strina I, et al. High outcome predictability after IVF using a combined score for zygote und embryo morphology und growth rate. Hum Reprod 2002; 17:2402–2409.
122. Nagy ZP, Dozortsev D, Diamond M, et al. Pronuclear morphology evaluation of embryo morphology significantly increases implantation rates. Fertil Steril 2003; 80:67–74.
123. Lan KC, Huang FJ, Lin YC, et al. The predictive value of using a combined Z-score and day 3 embryo morphology score in the assessment of embryo survival on day 5. Hum Reprod 2003; 18:1299–1306.

13

Embryo Culture Systems

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INTRODUCTION

The success of clinical IVF was initially compromised by sub-optimal culture conditions, resulting in impaired embryo development (1–6) and a subsequent loss of viability. However, research during the past 10–15 years has resulted in the development of more physiological and effective culture media capable of maintaining the viability of the developing embryo (7–10). This in turn has resulted in an increase in implantation rates and a decrease in the number of pregnancies lost. Furthermore, more suitable culture conditions produce embryos more able to survive cryopreservation (11). Therefore, improvements in embryo culture technology have significantly contributed to the increase in the overall success rates of human assisted conception.

In this chapter, the role of embryo culture systems and their individual components are analyzed along with the more recent development of multi-step culture systems. It is envisaged that, after reading such work on embryo culture, readers will be able to make informed decisions on the type of culture system most suited for their clinical requirements.

Types of Media for Embryo Culture

Culture media employed for clinical IVF vary greatly in their composition, yet there appears to be little difference between media in their ability to support development of the human embryo in vitro for up to 48 hours or in subsequent pregnancy rates after transfer (12). This has led to a great deal of confusion concerning the formulation of embryo culture media and the role of individual components in embryo development. An understanding of the role of culture media and their components has been hampered by the routine inclusion of serum in human embryo culture media. Serum has the ability to both mask potential embryo toxins and suppress the beneficial effects of other medium components. In light of this, there has been considerable research into the development of serum-free embryo culture media. Such studies have been invaluable in our understanding of the embryo's requirements during the preimplantation period.

Media used to culture the mammalian preimplantation embryo generally fall into one of four types.

Simple Salt Solutions with Added Energy Substrates

These media were originally formulated to support the development of zygotes from certain inbred strains of mice and their F1 hybrids (13). Examples of this type of media used in clinical IVF are M16 (14), T6 (14), Earle's (15), CZB (16), and KSOM (17). Derived from such types of media were human tubal fluid (HTF) medium (18,19), and P1 (20). As shown in Table 1 (21–25), there has been little change in the formulation of these media over the past 30 years. Such "simple" media are usually supplemented with either whole serum or serum albumin, and are used for the cleavage stage embryo only, i.e., pronucleate oocyte to the 8-cell stage.

Complex Tissue Culture Media

These media are commercially available and are designed to support the growth of somatic cells in culture, e.g., Ham's F-10 (Table 2) (26). Such media are far more complex, containing amino acids, vitamins, nucleic acid precursors, and transitional metals, and are usually supplemented with 5–20% serum. Importantly, such media were not formulated with the specific needs of the human embryo in mind, and they contain components which are now known to be detrimental to the developing embryo.

Simplex Optimized Media

This approach to formulate culture media depended on a computer program to generate successive media formulations based on the response of mouse embryos in culture (24,25). Once a specific medium was formulated, tested, and blastocyst development analyzed, the computer program would then generate several more media formulations for use in the next series of

Table 1 Composition (mM) of Simple Salt Solution with Added Energy Substrates used in Embryo Culture

Component	Whitten (1957) (21)	Brinster (1965) (22)	Whitten and Biggers (1968) (13)	M16 (1971) (14)	Earle's[a] (1971) (15)	HTF[a] (1981) (18)	CZB (1985) (16)	MTF[d] (1989) (23)	KSOM (1993) (24,25)	Basal XI HTF[a] (1995) (19)	P1[a] (1998) (20)
NaCl	118.46	119.23	68.49	94.66	116.30	101.60	81.62	114.19	95.00	97.6	101.6
KCl	4.74	4.78	4.78	4.78	5.36	4.69	4.83	4.78	2.50	4.69	4.69
KH_2PO_4	1.18	1.19	1.19	1.19	—	0.37	1.18	1.19	0.35	—	—
NaH_2PO_4	—	—	—	—	1.02	—	—	—	—	—	—
$CaCl_2.2H_2O$	—	1.71	—	1.71	1.80	2.04	1.70	1.71	1.71	2.04	2.04
$MgSO_4.7H_2O$	1.18	1.19	1.19	1.19	0.81	0.20	1.18	1.19	0.20	0.20	0.20
$NaHCO_3$	24.88	25.00	25.07	25.00	26.18	25.00	25.12	25.00	25.00	25.00	25.00
Ca Lactate	2.54	—	1.71	—	—	—	—	—	—	—	—
Na Lactate (D/L)	—	25.00	21.58	23.28	—	21.40	31.30	4.79	10.00[e]	21.4	21.4
Na Pyruvate	—	0.25	0.33	0.33	0.10	0.33	0.27	0.37	0.20	0.33	0.33
Glucose	5.55	—	5.56	5.56	5.55	2.78	—	3.40	0.20	—	—
BSA (mg/mL)	1.00	1.00	4.00	4.00	b	5.00	5.00	4.00	1.00	b	c
Ratios											
Na/K	24.21	28.39	19.34	24.00	26.79	29.26	23.01	24.18	45.68	30.71	30.71
Ca/Mg	2.15	1.44	1.44	1.44	2.22	10.02	1.44	1.44	8.55	10.2	10.2
L/P	—	100	70.58	70.55	—	64.85	115.93	12.95	50.00	64.85	64.85

Note: CZB contains 110 µM EDTA, 1.0 mM glutamine, and 5.5 mM glucose after 48 hours of culture from the zygote stage. KSOM contains 10 µM EDTA and 1.0 mM glutamine. Basal XI HTF contains 100 µM EDTA and 1.0 mM glutamine. P1 contains 50 µM taurine and 0.5 µM citrate. Penicillin (100 U/mL) and streptomycin present (50 µg/mL). Gentamycin present at 10 µg/mL.

[a]Used in clinical IVF.

[b]Medium supplemented with human serum albumin.

[c]Medium supplemented with synthetic serum substitute.

[d]Modifications to these media have included the addition of specific groups of amino acids resulting in significant improvements to mouse zygote development in culture.

[e]present as L-Lactate.

Abbreviations: HTF, human tubal fluid; CZB, Chatot, Ziomek and Bavister; MTF, mouse tubal fluid; KSOM, potassium simplex optimized medium; EDTA, ethylenediaminetetraceticacid; IVF, in vitro fertilization.

Table 2 Composition of Ham's F-10 Medium

Component	Concentration (mM)
NaCl	126.60
KCl	3.82
$MgSO_4 \cdot 7H_2O$	0.62
Na_2HPO_4	1.31
KH_2PO_4	0.61
$NaHCO_3$	14.28
$CaCl_2 \cdot 2H_2O$	0.30
$CuSO_4 \cdot 5H_2O$	0.00001
$FeSO_4 \cdot 7H_2O$	0.0030
$ZnSO_4 \cdot 7H_2O$	0.0001
Phenol Red	0.034
Sodium Pyruvate	1.00
Calcium Lactate	1.00
Glucose	6.11
Alanine	0.10
Arginine	1.21
Asparagine	0.11
Aspartic acid	0.10
Cysteine	0.26
Glutamate	0.1
Glutamine	1.0
Glycine	0.1
Histidine	0.14
Isoleucine	0.02
Leucine	0.10
Lysine	0.20
Methionine	0.03
Phenylalanine	0.03
Proline	0.10
Serine	0.10
Threonine	0.03
Tryptophan	0.003
Tyrosine	0.12
Valine	0.03
Biotin	0.0001
Ca pantothenate	0.0015
Choline chloride	0.005
Cyanocobalamine	0.001
Folic acid	0.003
Inositol	0.003
Nicotinamide	0.005
Pyridoxine	0.001

(*Continued*)

Table 2 Composition of Ham's F-10 Medium (*Continued*)

Component	Concentration (mM)
Riboflavin	0.001
Thiamine	0.003
Hypoxanthine	0.03
Lipoic acid	0.001
Thymidine	3.00

Note: Penicillin present at 100 U/mL. Streptomycin present at 50 μg/mL. Modifications as per the Center for Reproductive Medicine.

cultures. This procedure was performed several times to generate media that supported high rates of blastocyst development of embryos derived from the oocytes of outbred mice (CF1) crossed with the sperm of an F1 hybrid male, and were termed SOM and KSOM. Such media were subsequently modified by another laboratory to include amino acids (KSOMAA) (27). This last phase of medium development was based on previous studies on the mouse embryo (28) and did not involve the simplex procedure. This single medium formulation, KSOMAA, has been used to produce human blastocysts in culture (29). In such types of media, the embryo therefore has to adapt to its surroundings as it develops and differentiates.

Sequential Media

The approach taken in our laboratory has not only been to learn from the environment to which embryos are exposed in vivo (23,30), but also to study the physiology and metabolism of the embryo in culture, in order to determine what causes intracellular stress to the embryo (7,9,31–36). By being able to identify and monitor such stress, we have been able to develop stage specific culture media that substantially reduce culture-induced trauma. The development and characterization of such sequential media has been published in detail elsewhere (37–39).

Examples of sequential media include G1/G2 (Table 3) (37,40,41), universal IVF medium and M3 (42), and P1 together with blastocyst medium (43). Interestingly, medium M3 is a modification of Ham's F-10 and F-12, while blastocyst medium is a mosdification of Ham's F-10.

COMPOSITION OF EMBRYO CULTURE MEDIA

The composition of embryo culture systems can be broken down into the following components:

- Water
- Ions

Table 3 Composition of a Sequential Medium

	Concentration (mM)	
Component	G1.2	G2.2
NaCl	90.08	90.08
KCl	5.5	5.5
Na_2HPO_4	0.25	0.25
$MgSO_4 \cdot 7H_2O$	1.0	1.0
$CaCl_2 \cdot 2H_2O$	1.8	1.8
$NaHCO_3$	25.0	25.0
Sodium pyruvate	0.32	0.10
Sodium lactate (L)	10.5	5.87
Glucose	0.5	3.15
Alanine	0.1	0.1
Aspartic acid	0.1	0.1
Asparagine	0.1	0.1
Arginine	—	0.6
Cystine	—	0.1
Glutamate	0.1	0.1
Alanyl-glutamine	1.0	0.5
Glycine	0.1	0.1
Histidine	—	0.2
Isoleucine	—	0.4
Leucine	—	0.4
Lysine	—	0.4
Methionine	—	0.1
Phenylalanine	—	0.2
Proline	0.1	0.1
Serine	0.1	0.1
Taurine	0.1	—
Threonine	—	0.4
Tryptophan	—	0.05
Tyrosine	—	0.2
Valine	—	0.4
Choline chloride	—	0.0072
Folic acid	—	0.0023
Inositol	—	0.01
Nicotinamide	—	0.0082
Pantothenate	—	0.0042
Pyridoxal	—	0.0049
Riboflavin	—	0.00027
Thiamine	—	0.00296
EDTA	0.01	0.00
HSA	5 mg/mL	5 mg/mL

Penicillin present at 100 U/mL.
Abbreviations: EDTA, ethylenediaminetetraacetic acid; HSA: human serum albumin.
Source: From Ref. 40.

- Carbohydrates
- Amino Acids
- Vitamins
- Nucleic Acid Precursors
- Chelators
- Antioxidants
- Antibiotics
- Protein/macromolecules
- Hormones and growth factors
- Buffer system

The role of each component on embryo development in culture, with focus on the pre- and post-compaction stages, will be discussed in turn.

Water

Water is the major component of any medium, making up around 99% of the contents. The source and purity of water used for media preparation is, therefore, a major factor in assuring the quality of media. The ability of embryos to develop in culture is positively correlated to water quality. Whittingham (14) demonstrated that the development of 2-cell mouse embryos to the blastocyst in culture was enhanced when the media was prepared using triple distilled water as opposed to double or single distilled water. However, the process of distillation has inherent problems due to the possible leaching of ions and pyrogens from the glassware. A more reliable water purification system is ultrafiltration, which produces pyrogen-free water with a resistance >18 megOhms. Depending upon the local water source however, it may be required to distill or pre-filter the original supply before processing. An alternative to in-house water preparation is commercially available high quality water, which should come endotoxin-tested and contain endotoxin levels less than 0.1 IU/mL.

Ions

The ionic basis of culture media used for clinical IVF varies markedly (Table 4). Surprisingly, relatively little is known about the role of ions during preimplantation embryo development. The ionic composition of oviduct fluid from the human and mouse has been sampled by micropuncture and analyzed using an electron probe (Table 4) (30,44–46). Mammalian oviduct fluid is characterized by high potassium and chloride concentrations and a high overall osmolality (44,45). Interestingly, high osmolality balanced salt solutions with added carbohydrates as energy sources do not support high levels of embryo development in vitro (47,48).

Optimization of the ionic component of media has been compounded by the ability of embryos from certain strains of mice to develop apparently normally in culture to the blastocyst stage in a wide range of ion concentrations.

Table 4 Concentration (mM) of Ions, Carbohydrates, and Glutamine in Mammalian Fluids and Embryo Culture Media

Component	Human oviduct fluid [a](30,44)	Human uterine fluid [a](30)	Human serum (44)	Mouse oviduct fluid (23,45)	HTF medium (18)	Ham's F-10 (26)	Menezo's B2/3 (46)	KSOM (17,25)	XI (19)	G1 (40)	G2 (40)
Na	130	nd	145	139	148	143	129	130.2	144.3	126.4	121.55
Cl	132	nd	nd	165	110	131	114	106.4	106.4	99.2	99.2
K	21.2	nd	5.0	23.4	5.1	4.4	9.8	2.85	4.70	5.50	5.50
Ca	1.13	nd	1.13	1.71	2.04	0.30	0.56	1.71	2.04	1.80	1.80
Mg	1.42	nd	2.00	1.04	0.20	0.62	0.81	0.2	0.2	1.0	1.0
S	12.3	nd	nd	8.45	0.20	0.62	0.17	0.2	0.2	1.0	1.0
P	8.69	nd	nd	8.93	0.37	1.92	0.90	0.35	—	0.25	0.25
Pyruvate	0.32	0.10	0.10	0.37	0.33	1.00	2.27	0.20	0.33	0.32	0.10
L-Lactate	10.50	5.87	0.60	4.79	—	—	—	10.0	—	10.50	5.87
D/L-Lactate	—	—	—	—	21.4	2.23	0.56	—	21.4	—	—
Glucose	0.50	3.15	5.00	3.40	2.78	6.11	6.67	0.2	0.00	0.50	3.15
Glutamine	0.30	nd	nd	0.20	0.00	0.30	0.17	1.0	1.0	1.0[b]	0.5[b]
Ratios											
Na/K	6.1	—	29.0	5.9	29.0	32.3	13.1	45.6	30.7	22.98	22.1
Ca/Mg	0.80	—	0.57	1.64	10.10	0.48	0.69	8.56	10.2	1.8	1.8
L/P	8.25	25.22	6.00	12.95	64.85	0.30	0.25	50.0	64.85	31.81	58.7

[a]Mid-cycle.
[b]Present as alanyl-glutamine.
Abbreviations: HTF, human tubal fluid; KSOM, potassium simplex optimized medium.

However, the suitability of using in vitro development to the blastocyst stage as the sole criterion for assessing the suitability or otherwise of a culture medium is highly questionable (49,50). The only true test of a medium's suitability is to transfer embryos to recipient females and quantify fetal development. Unfortunately, however, there is relatively little information available regarding embryo viability in animal models, and so almost all data has come from in vitro studies. Wales (51) used the development of 2-cell mouse embryos to the blastocyst in order to determine the range of ion concentrations capable of supporting development in vitro. Embryos formed blastocysts in medium with a potassium concentration ranging between 0.4 and 48 mM, a magnesium concentration between 0 mM and 9.6 mM, a calcium concentration between 0.1 mM and 10.2 mM, and a phosphate concentration between 0 mM and 7.2 mM, with a narrow range of optima for all ions. Studies on the hamster have also shown that the first cleavage and development of 2-cell embryos to the blastocyst occur in a wide range of sodium, magnesium, calcium, and potassium concentrations (52,53). Unfortunately, it is difficult to interpret the effects of individual ions on embryo development and viability, as there are many subtle interactions which exist between ions, carbohydrates, and amino acids (see below).

High potassium levels in culture media have been reported to have a beneficial effect on sperm capacitation (54) and embryo development in vitro (51,55,56). However, there is conflicting data on the positive effects of potassium on embryo development (49,57,58). The interaction of ions with other medium components must therefore be taken into account.

High concentrations of NaCl (125 mM) in culture media are detrimental to mouse embryo development to the blastocyst in vitro (17,48). Reducing the sodium chloride concentration to 85 mM in the medium increases the rates of both mRNA (27) and protein (59) synthesis of cleavage stage mouse embryos in vitro.

Studies on the effect of magnesium and calcium in the medium for the development of 2-cell mouse embryos in culture determined that magnesium was not essential for development to the blastocyst stage; however, calcium is essential for embryos to undergo compaction in vitro (51,60). More recently, the effects of extracellular magnesium and calcium levels on the ability of early embryos to regulate intracellular homeostasis have been examined. Early hamster embryos up to six hours following fertilization have a reduced ability to regulate intracellular calcium levels. This is exacerbated by low magnesium:calcium ratios in the medium (61,62). This reduced ability of embryos to regulate ionic homeostasis is directly related to the loss in viability (62) and increased calcium mobilization is reported to alter levels of gene expression (63). Interestingly, the appearance of the appropriate transporter systems in the hamster embryo correlates with the dispersion of the cumulus cells, i.e., prior to this time the cumulus cells may have a protective action. Therefore, the premature removal of cumulus cells in an ICSI

procedure may render the oocyte susceptible to ionic stress. The ionic composition of the culture medium is an important consideration as external ion concentrations can have a profound effect on intracellular ion levels, and therefore the regulation of normal cellular processes.

There has been much discussion in the literature in recent years regarding the rationale of phosphate inclusion in embryo culture medium. In a simple culture medium containing glucose such as HTF or Earle's balanced salts, the presence of phosphate resulted in retarded human embryo development (19). Interestingly, phosphate is only inhibitory (with the exception of the hamster 2-cell embryo) in the presence of glucose, the mechanism of which is discussed in detail below. However, when phosphate is present in more physiologically defined media, i.e., in the presence of specific amino acids, it does not have an inhibitory effect. Such observations are consistent with phosphate being present in the fluids of the human female reproductive tract (44), confirming the artifactual nature of phosphate's detrimental effects in culture. Furthermore, it is consistent that at later stages of development, when the cells of the embryo begin to take on a more somatic cell like physiology, phosphate is beneficial (64).

Further to their specific functions, the ions in any medium make the largest single contribution to osmotic pressure. The optimal osmolality for the development of human embryos in culture has not been determined. However, mouse (65) and hamster (52) embryos will develop in a wide range of osmolalities (200–350 mOsm). Although conventional embryo culture media has an osmolality of between 275 and 295 mOsm, enhanced development of mouse embryos appears to occur at reduced osmolalities (13,17). Again, however, it is important to note that such studies were performed using simple embryo culture media, i.e., balanced salt solutions, in the absence of amino acids. It is now evident that the inclusion of osmolytes, such as betaine, or specific amino acids, such as glycine, in the culture medium can reduce any osmotic stress (35,36,47,48,66,67), thereby allowing apparently normal embryo development to occur over a wider range of osmotic pressures and ion concentrations.

Carbohydrates

Carbohydrates are present within the luminal fluids of the female reproductive tract. Their levels vary both between the oviduct and uterus and within the cycle (30,68). Therefore, the developing embryo is exposed to gradients of carbohydrates as it develops (Table 4). Together with amino acids, carbohydrates are the main energy substrates for the embryo. Most embryo culture media contain the carbohydrates pyruvate, lactate, and glucose. If one or more of these nutrients are absent from the medium formulation, then they are frequently added in low concentrations when serum is used to supplement the media. Furthermore, the cumulus cells surrounding the oocyte and early embryo readily produce both pyruvate and lactate from

glucose (23,30,69). The levels of carbohydrates in the fluid of the mammalian female tract and a variety of culture media are presented in Table 4.

The precise substrate requirements for the human embryo have yet to be fully elucidated. However, analysis of carbohydrate uptakes in vitro has revealed that the human embryo has an initial preference for pyruvate (70–73), whilst glucose uptake increases with development (71,72,74). Such data indicate that glucose is not utilized as a major energy source by the early embryo. This pattern of carbohydrate utilization has been reported for other mammalian species. Mouse and sheep oocytes and zygotes take up little glucose compared to pyruvate. Around the time of compaction there is a switch in carbohydrate uptake and metabolism (75–77). Such studies on nutrient uptake reflect the findings of earlier culture experiments which found that the mouse oocyte and zygote exhibit an absolute requirement for pyruvate as an energy source (78). The omission of pyruvate from the medium for the development of the human embryo results in 84% of embryos arresting development at, or prior to, the 8-cell stage. Pyruvate as the sole energy substrate is also able to support the development of human zygotes to the blastocyst stage (79). It has, therefore, become dogma over the years that the first cleavage division of the mouse embryo is dependent upon the presence of pyruvate in the culture medium (78). However, recent research has shown that in the presence of aspartate and lactate, there exists sufficient activity of the malate–aspartate shuttle in the embryo's mitochondria to overcome this dependence on pyruvate. Indeed, viable mouse blastocysts can be obtained from zygotes cultured in the complete absence of pyruvate (80).

Interestingly in the mouse embryo, lactate can be utilized as an energy source from the 2-cell stage and acts synergistically with pyruvate (81). There have been conflicting studies on the optimal concentration of lactate in the culture medium to support mouse embryo development to the blastocyst stage. Cross and Brinster (81) reported that a lactate concentration of 30 mM is optimal to support zygote development to the blastocyst, while other studies have reported 10 mM to be optimal (82,83). A subsequent study showed that mouse zygotes cultured to the 8-cell stage in the presence of high lactate concentration (20 mM) were more viable than embryos cultured in a low lactate (4.79 mM) concentration (49). However, when the culture period was extended to the morula stage prior to transfer, the reverse was true with viability increased by culture in lower lactate. Significantly, the regulation of metabolism of these carboxylic acids changes with development, which highlights the physiological differences between the zygote and blastocyst stages (34).

An important point to note is that, in almost all embryo culture media, lactate is present as a 50:50 mixture of both the D- and L-isomer in sodium lactate syrup. As only the L-isomer is biologically active, the effective lactate concentration in embryo culture media is half of that given in the formulation. Lactate is a weak acid which readily enters the embryo

and at concentrations of 5 mM or greater induces a significant drop in intracellular pH (84). Therefore, it is recommended that sodium lactate salt be used in culture medium preparation in order to avoid the presence of excess lactate present as the D-isomer which, although not biologically active, can still induce a fall in pHi and therefore affect cellular physiology.

Glucose as the sole substrate cannot support mouse embryo development prior to the late 4-/early 8-cell stage (22,85). This inability to utilize glucose as an energy source during the first three cell cycles has been attributed to a blockade in glycolysis (86–88). Studies on the mouse (16), hamster (89), sheep (90), cattle (91–93), and human (19,79) have all demonstrated that glucose in the presence of phosphate is responsible for the retardation or developmental arrest of cleavage stage embryos in culture. This has been attributed to the premature stimulation of glycolysis, a phenomenon similar to the Crabtree effect (7,94–98). The Crabtree effect, as described in tumor cells in culture, depends on the continued activity of hexokinase in the presence of increasing product, glucose-6-phosphate. The isozyme of hexokinase present in these cells must, therefore, be a form of the enzyme that is not completely inhibited by glucose-6-phosphate. Kinetic analysis of hexokinase in preimplantation mouse embryos indicates that there is a switch from isozyme I at the zygote to isozyme II at the blastocyst stage (99). Indeed, these two isoforms have differing sensitivities to phosphate, with the inhibition of isozyme I by glucose-6-phosphate being overcome by phosphate (100). It is possible that the differences in the ability to regulate metabolism in culture media lacking amino acids by the early cleavage stage embryo and the blastocyst stage may be due to differences in the isozyme of glycolytic enzymes, such as hexokinase (7). Utilization of glycolysis is at the expense of oxidative metabolism and could result in impaired energy production (94). However, this inhibition of glucose can be alleviated by the inclusion of amino acids (38,96,101,102), EDTA (7,103), and vitamins (32), highlighting the interactions which exist between medium components and the potential hazards of using simple salt solutions for embryo culture. In light of the potential toxicity of glucose in such media as HTF, it has been advocated to remove it from embryo culture media (19,20,104). Such a course of action may work for the culture of the cleavage stage embryo, but the removal of glucose from medium used for blastocyst culture results in a significant reduction in subsequent fetal development, highlighting its intrinsic role in the development of a viable embryo (105,106). Indeed, the removal of glucose from a culture medium can be considered as alleviating a culture-induced artifact by the introduction of a second artifact, i.e., the removal of glucose from the culture medium when it is present in both oviduct and uterine fluids (30), and when the oocyte and embryo have a specific carrier for this hexose (107–110). The reasons for the inclusion of glucose in embryo culture media are, therefore, not only is it required for energy production but it is also essential for biosynthesis. The metabolism of glucose

through the pentose phosphate pathway not only generates NADPH required for lipid/membrane biosynthesis, but generates ribose moieties for nucleic acid and triacylglycerol biosynthesis. Furthermore, at the time of implantation, the environment around the blastocyst is relatively anoxic (111,112). This means that glycolysis may well be the only means of generating energy before angiogenesis in the endometrium is complete (98,113). A source of glucose for glycolysis could be the embryo's own glycogen stores. Should the embryo have prematurely used such glucose stores during development because there was no glucose present in the culture medium, then the embryo will have a reduced ability to implant. Indeed, mouse blastocysts in culture which exhibit excessive lactate production from their endogenous energy reserves have a significantly reduced developmental potential after transfer (114).

Interestingly, glucose is used by sperm and is therefore often present in the insemination medium. However, in all likelihood the oocyte and early zygote are not exposed to high glucose levels as the surrounding cumulus cells readily metabolize it to pyruvate and lactate (30).

In conclusion, the preimplantation embryo undergoes a switch in carbohydrate utilization during development. Initially pyruvate and actate are the preferred nutrients, with glucose utilization significantly increasing post-compaction. Such changes in utilization mirror the availability of carbohydrates within the female tract. Pyruvate and lactate are at their highest concentration within the human fallopian tube, while glucose is at its lowest level. In contrast, within the uterus, pyruvate and lactate concentrations are at their lowest and glucose at its highest (Table 4) (30), with the presence of glucose ensuring the viability of the developing blastocyst.

Amino Acids

Oviduct and uterine fluids contain significant levels of free amino acids (23,115–118). Oocytes and embryos possess specific transport systems for amino acids (119) and maintain an endogenous pool of amino acids (120). Indeed, amino acids are readily taken up and metabolized by the embryo (121,122). Such data support the notion that amino acids have a physiological role in the pre- and peri-implantation period of mammalian embryo development.

Oviduct and uterine fluids are characterized by high concentrations of the amino acids alanine, aspartate, glutamate, glycine, serine, and taurine (116–118). With the exception of taurine, the amino acids at high concentrations in oviduct fluid bear a striking homology to those amino acids present in Eagle's (Table 5) (123) non-essential amino acids. Analysis of human and mouse oviduct fluids have also demonstrated that there are significant levels of glutamine present (Table 4). Studies on the embryos of several mammalian species, such as mouse (28,105,124,125), hamster (8,66,126,127),

Table 5 Composition of Eagle's Amino Acids (mM)

Non-essential	
Alanine	0.1
Asparagine	0.1
Aspartate	0.1
Glycine	0.1
Glutamate	0.1
Proline	0.1
Serine	0.1
Essential	
Arginine	0.6
Cystine	0.1
Glutamine	2.0[a]
Histidine	0.2
Isoleucine	0.4
Leucine	0.4
Lysine	0.4
Methionine	0.1
Phenylalanine	0.2
Threonine	0.4
Tryptophan	0.05
Tyrosine	0.2
Valine	0.4

[a]Glutamine is frequently used at a concentration of 0.5–1 mM in embryo culture.
Source: From Ref. 123.

sheep (102,128), and cows (37,91), have all demonstrated that the inclusion of specific amino acids in the culture medium enhances embryo development to the blastocyst stage. In the mouse embryo it has been determined that inclusion of non-essential amino acids and glutamine in the medium significantly increases the rate of zygote development to the blastocyst in culture, and indeed can alleviate the 2-cell block (105). Non-essential amino acids and glutamine stimulate cleavage rates (28,129,130), blastocyst formation and hatching of cultured mouse embryos (Table 6) (28,50,130). Significantly, the inclusion of amino acids in the culture medium is associated with the production of mouse blastocysts in vitro at the same time as they would form in vivo (40,131). It has been demonstrated that even a transient exposure (<5 minute) of mouse zygotes to medium lacking amino acids impairs subsequent developmental potential (Fig. 1) (105). It has subsequently been shown that during this 5 minute period in a simple medium lacking amino acids, the embryo loses its entire endogenous pool, which takes several hours of active transport to replenish after returning the

Table 6 Effect of Amino Acids on Development of Mouse Zygotes Cultured for 72 hours

Amino acids	Stage of development reached (%)					
	< Morula	Morula	Early blastocyst	Expanded blastocyst	Hatching blastocyst	Total blastocyst
None	0	78[a]	22	0	0	22[a]
Non-essential	6	6[a,b]	18	36	34	88[a,b]
Essential	8	70[b]	18	4	0	22[b]

Note: Base medium was modified mouse tubal fluid medium.
[a, b]Like pairs are significantly different: $P < 0.01$.
Source: From Ref. 28.

embryo to medium with amino acids (Baltz JM. Personal Communications. 1998). This has implications for the collection of oocytes, and more importantly the manipulation of denuded oocytes during ICSI, where plausibly the inclusion of amino acids in the holding medium will decrease or prevent

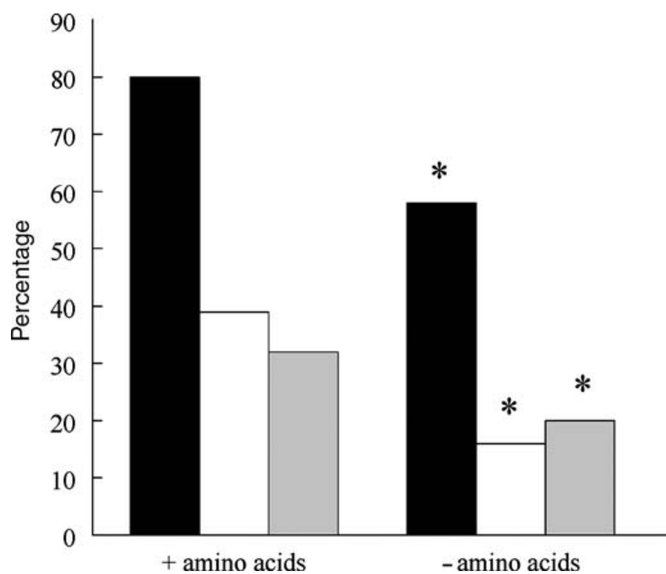

Figure 1 Effect of collection of CF1 mouse zygotes in medium without amino acids on subsequent development. Zygotes were collected in medium either containing non-essential amino acids and glutamine or in the same medium without the amino acids. Embryos were in the collection medium for less than five minutes. Solid bars represent morula/blastocyst development. Open bars represent blastocyst development. Shaded bars represent blastocyst hatching. *Significantly reduced compared to collection with amino acids ($P < 0.05$). *Source*: From Ref. 105.

intracellular stress. Furthermore, it highlights the significant physiological role of cumulus cells preventing homeostatic stress in vitro.

While addition of non-essential amino acids to the culture medium increased cell numbers at the blastocyst stage, the increase in cleavage rate was attributed solely to an increase in zygote cleavage rate up to the 8-cell stage (50,129,130). After compaction, non-essential amino acids and glutamine stimulate cleavage of the trophectoderm and increase blastocoel formation and hatching (50). In contrast, essential amino acids which are at low concentrations in the oviduct reduce the cell number of blastocysts from cultured zygotes (28,50). This inhibition of development can be attributed to the negative effect of essential amino acids at the concentration present in tissue culture media during the first four cell cycles of development (132). Interestingly however, after the 8-cell stage essential amino acids stimulate cleavage rates and increase development of the inner cell mass (ICM) in the blastocyst (50). Studies on the development of single amino acids on hamster embryos by Bavister and co-workers (66,127,133) found that asparagine, aspartate, glycine, histidine, serine and taurine stimulated hamster zygote development to the blastocyst in culture, whilst cysteine, isoleucine, leucine, phenylalanine, threonine, and valine were inhibitory. All the inhibitory amino acids are present in Eagle's essential amino acids, whilst the stimulatory amino acids to hamster embryo development other than histidine are found in Eagle's non-essential amino acids (Table 5). Evidently, the term essential and non-essential have little meaning with regard to embryology, they have served as convenient groupings in the initial analysis of amino acids (28). Other terms, such as "cleavage amino acids" and "ICM amino acids" may be functionally more relevant (134). As research progresses, other amino acids may be added to such groupings.

The beneficial effects of Eagle's non-essential amino acids and glutamine on early embryo development have been proposed to come from their use not solely as energy substrates, but rather as intracellular osmolytes (47,67,135) and regulators of intracellular pH (31). The use of amino acids as intracellular regulators is common amongst unicellular organisms and their use by the pre-compacted embryo may well stem from the simplistic organization of individual cells within the embryo. Prior to compaction, each cell is in direct contact with the external medium, while in post-compaction the embryo has a transporting epithelium and can therefore actively regulate its internal environment. In support of this hypothesis is the observation that the beneficial effects of betaine and glycine in medium containing a high sodium concentration is restricted to stages prior to compaction (35). Furthermore, it has been demonstrated that the formation of a transporting epithelium at compaction marks the ability of the embryo to regulate against an acid load (31).

Most importantly, amino acids have been reported to increase viability of cultured embryos from several species after transfer to recipients

(37,49,102,136,137) as well as increasing embryo development in culture. In the mouse, culture with those amino acids present at high levels in the oviduct to the 6–8 cell stage prior to transfer significantly increased implantation rates and fetal development after transfer (130). In contrast, embryos cultured to the morula or blastocyst stage prior to transfer had greatest fetal development rates after culture with all 20 amino acids, confirming that the pre-implantation embryo undergoes a switch in amino acid requirements as development proceeds from the zygote to the blastocyst stage. Furthermore, mouse zygotes cultured to the blastocyst stage in the appropriate sequential media are able to implant at equivalent rates to in vivo developed blastocysts (40,50,131).

Such data therefore supports the notion that amino acids should be included in human embryo culture media. Indeed, recently it has been demonstrated that the addition of glutamine to a simple culture medium significantly increases the development of human blastocysts in culture and subsequent pregnancy rates (138). It is also evident that optimal development in culture requires the presence of different groups of amino acids. To this end, sequential media designed to support the development of the human blastocyst in culture, G1 (pre-compaction) (37) and G2 (post-compaction) (40) use the amino acids that stimulate cleavage stages for development prior to compaction, and includes all of the amino acids that stimulate blastocyst formation and ICM development for development post-compaction (37,40).

Ammonium

Research on the role of amino acids in mammalian embryo development is very exciting. However, there is a negative effect associated with the use of amino acids in culture, which acts as a timely reminder that one is dealing with artificial conditions which are not identical to those in the female tract. The following "Catch 22" is in no way an isolated example of the problems that are encountered in embryo culture. Within the female tract's, changing environment the embryo is exposed to a constantly which is altered by the transporting epithelial cells lining the tract. The problem with amino acids in culture media is that although they do regulate embryo development, they are both metabolized by embryos to produce ammonia and more importantly they spontaneously breakdown at 37°C and release ammonia (Fig. 2). This results in the build up of embryo-toxic ammonium ions in the medium, a phenomenon which does not occur within the dynamic environment of the tract, as levels of ammonium are close to zero in oviduct fluid (116,139,140). Ammonium ions in the medium can not only alter embryo differentiation, metabolism, and gene expression in vitro (141,142), but also significantly reduce implantation and fetal development rates after transfer (130). Furthermore, in the mouse a significant proportion of fetuses resulting from embryos cultured in the presence of toxic concentrations of

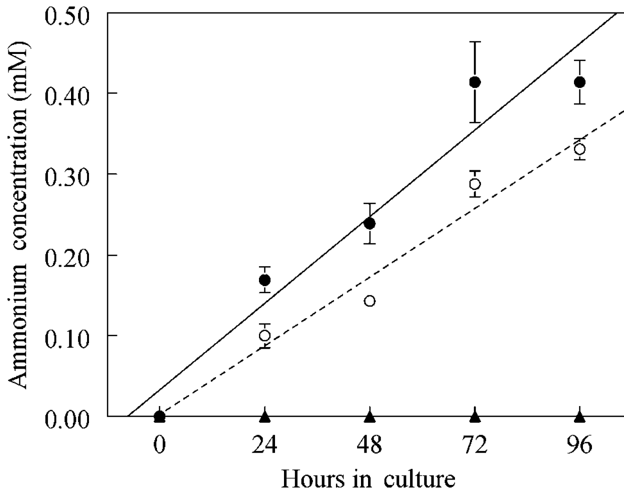

Figure 2 Ammonium production in culture media incubated at 37°C. Levels of ammonium production were examined in medium without amino acids (closed triangles), medium containing amino acids (open circles), and in culture medium containing amino acids and mouse embryos (closed circles). There was no detectable ammonium production in the medium without amino acids. Ammonium production increased linearly over time in the media containing amino acids. The level of ammonium production was increased further when the embryos were also in the culture drop. *Source*: From Ref. 28.

ammonium exhibited the birth defect exencephaly (130,143). Of all amino acids, glutamine is the most labile. The substitution of this amino acid with the more stable alanyl-glutamine, significantly reduces the generation of ammonium in the culture medium. The detrimental effects of ammonium on embryo development in vitro and in vivo after transfer can be alleviated by the renewal of the culture medium after 48 hours of culture. Therefore, if amino acids are included in the culture medium formulation, it is imperative that media is not stored at 37°C and that the culture period does not extend beyond 48 hours before embryos are transferred to fresh medium, because birth defects can still be induced by the prolonged exposure of embryos to amino acids, even in the absence of glutamine (144). Significantly, it has now been reported that increasing concentrations of ammonium in the culture medium have a negative impact on human blastocyst development (145).

Vitamins

Although vitamins are present in complex media formulations, their effects on embryo development remain largely unknown. while both human (146,147) and mouse zygotes (13) will form blastocysts in culture in the absence of vitamins, the rabbit blastocyst requires vitamins for blastocoel

expansion (148). However, Kane (148) found that B12, one of the vitamins present in the tissue culture medium Ham's F-10, caused a decrease in blastocyst expansion. Furthermore, a distinction should be made between the type of blastocyst formed by the rabbit compared to that of the human and mouse. The rabbit blastocyst undergoes prolific expansion, whilst the volume of the human and mouse blastocyst are only slightly larger than that of the oocyte. In the mouse, culture of zygotes to the blastocyst stage has been shown to be inhibited by the water-soluble vitamins present in both Ham's F-10 medium and MEM (149). Specifically, nicotinamide inhibited blastocyst cell number in vitro and reduced viability after transfer (149). Interestingly, however, the vitamins present in MEM had no detrimental effect on mouse zygote development to the blastocyst stage when amino acids were also present (49). These data further highlight the interactions that exist between various medium components. Importantly, vitamins and amino acids act in synergy to prevent perturbations in metabolism and loss of viability induced by sub-optimal culture conditions (Fig. 3) (7,32). As B-group vitamins are an integral part of carbohydrate and amino acid metabolism, certain vitamins may therefore have an important role to play in embryo development especially in the presence of amino acids. At present their function in human embryo development remains unclear, but

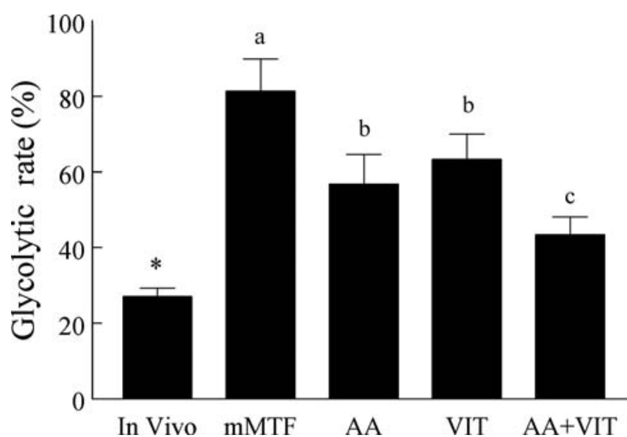

Figure 3 Effect of amino acids and vitamins on blastocyst metabolism. Mouse blastocysts developed in vivo have a low level of aerobic glycolysis. Culture for 6 hr in a simple medium lacking amino acids and vitamins (mMTF) results in an abnormal increase in glycolysis that is associated with reduced viability. Addition of amino acids or vitamins to the medium reduces this perturbation in metabolism. Amino acids and vitamins act in synergy to further reduce this increase in glycolysis. *Note*: [a–c]Different superscripts are significantly different ($P < 0.05$). [*]Significantly different to all culture treatments. *Source*: From Ref. 32.

a role in maintaining adequate levels of oxidation and subsequently blastocyst expansion and/or hatching cannot be excluded.

Nucleic Acid Precursors

The development of human and mouse zygotes to blastocysts in culture does not require the presence of nucleic acid precursors in the medium, although mouse embryos can incorporate exogenous radiolabeled nucleosides into their RNA and DNA (2). The ability of embryos to grow in the absence of nucleosides indicates that de novo pathways of nucleic acid synthesis are active at this stage. Loutradis et al. (150) observed that hypoxanthine, present in Ham's F-10, induced a block in mouse embryo development at the 2-cell stage in vitro. Hypoxanthine is thought to inhibit the purine salvage pathway (151). Ham's F-10 without hypoxanthine is available commercially. Subsequent studies have revealed that both adenosine and inosine are also detrimental to the development of mouse embryos after the first cleavage division (152). Without further research into the role of nucleotides in embryo development, their omission from embryo culture media formulations would seem advisable.

Chelators

The addition of chelators of heavy metal ions to culture media has been reported to enhance the development of pre-implantation embryos in vitro. Addition of EDTA to the culture media increases the development of mouse zygotes beyond the 2-cell stage and increases development to the blastocyst stage (16,24,105,153,154). However, the stimulatory effect of EDTA was only evident at concentrations between 10 μM and 150 μM, whereas a concentration of 200 μM inhibited development to the blastocyst (155). A recent report on the human embryo showed that the inclusion of EDTA to medium HTF without glucose and phosphate significantly increased the development of zygotes to the blastocyst stage in vitro (19). In light of these studies many new media formulated for the development of mammalian embryos in culture such as CZB (16), KSOM (17,25,56), G1 (37), and mHTF (19) now contain EDTA. A commercially available medium supplement, SSR2 from MediCult contains a chelation system of 4.3 μM EDTA together with 40 μM citrate, which appears to be a suitable chelation system based upon the stability of the iron chelates formed (156).

The beneficial effect of EDTA on embryo development in vitro has been isolated to the cleavage stage embryo (103,105,157). The presence of EDTA in the medium after compaction significantly reduces fetal development after transfer (38,105). It is therefore apparent that whilst EDTA stimulates development prior to compaction, the presence of EDTA for blastocyst development compromises the subsequent developmental competence of the embryos. Furthermore, in the cow, the presence of 100 μM

EDTA in the culture medium for development post-compaction specifically retarded development of the ICM (158). Interestingly, the ICM is dependent upon glycolysis for its energy production (159). Therefore, the detrimental effect of EDTA on ICM development may be explained by altered ICM energy production resulting in reduced fetal development, as ICM development is directly related with fetal development after blastocyst transfer (50). As analysis of the glycolytic enzyme 3-phosphoglycerate kinase in 2-cell, 8-cell and blastocyst stage embryos revealed that enzyme activity was significantly reduced by 10 μM EDTA as well as by 100 μM EDTA, it would better to err on the side of caution and not expose blastocysts to EDTA (103).

Another chelator of free metal ions, transferrin, has also been demonstrated to increase development of mouse zygotes through the 2-cell block to the blastocyst stage (160,161). It is proposed that transferrin increases embryo development by the chelation of ferric ion, thus preventing the formation of free oxygen radicals in the culture medium which cause oxidative stress to the embryo. However, it has been shown that in a medium containing EDTA and non-essential amino acids and glutamine, the inclusion of transferrin did not increase mouse embryo development to the blastocyst stage (105). Therefore in the presence of EDTA and amino acids, transferrin may not be essential.

Antioxidants

It has been proposed that one of the causes for the retarded development of preimplantation embryos in culture compared to those developed in vivo is oxidative stress. Potential sources of such stress include the use of high oxygen tension (i.e., 20%), exposure to light, and the presence of transitional metals in the culture medium (162). Therefore, several studies have examined the effect of known antioxidants on preimplantation embryo development, although the data to date remain rather contradictory. Supplementation of medium with superoxide dismutase (SOD), which dismutases superoxide radicals, increased the development of mouse zygotes beyond the 2-cell block to the blastocyst stage (163,164). However, several studies have reported that SOD had no effect on either mouse (165), rabbit (166) or bovine (167) embryo development in vitro.

Similarly, Legge and Sellens (168) reported that addition of glutathione to the medium stimulated development of mouse zygotes in culture, whereas Nasr-Esfahani and Johnson (169) reported that the addition of glutathione to the medium did not increase embryo development in culture. Glutathione is present in fluid of the reproductive tract and, therefore, may have a role in embryo development (170). Moreover, the beneficial effects of the addition of cysteamine to the medium for bovine (171–174) and pig (175) oocyte development have been attributed to an increase in intracellular glutathione levels (176). Therefore, it is feasible to suggest that the maintenance

of a high intracellular pool of glutathione may be important for high rates of development of the oocyte and early embryo.

The conflicting reports as to the benefits of adding antioxidants to culture media may in part be explained by their use in isolation and not as part of a more complete antioxidant system. For example, when SOD is present to dismutase superoxide radicals to hydrogen peroxide, then catalase and/or glutathione may be required to remove the peroxide formed. The presence of more than one antioxidant may facilitate the cycling of antioxidants back into the reduced forms. Alternatively, the generation of superoxide radicals will depend on the medium used for culture. Interestingly, however, it has been shown that pyruvate present in the culture medium is a powerful antioxidant (177) and readily decreases intracellular hydrogen peroxide levels within the embryo (178,179). As pyruvate is present in all media for embryo development, by default embryo culture media are supplemented with an antioxidant. Similarly, the amino acid taurine present in such media as G1 (38,40) and P1 (20), may also serve as an antioxidant (180). Finally, the addition of the water soluble antioxidant ascorbate has been shown to be highly beneficial when added to media used in slow freezing of embryos (181). Presumably, the presence of such an antioxidant is beneficial in reducing the impact of reactive oxygen intermediates generated during the freezing process.

Antibiotics

Traditionally, antibiotics such as penicillin, streptomycin, or gentamycin have been routinely included in embryo culture media. However, a recent study reported improved cleavage rates of human embryos in medium free of antibiotics, questioning the practice of routinely adding antibiotics to the culture medium (182). However, it is important to note that the washing of embryos in medium supplemented with antibiotics can remove any bacterial contamination (183) and this may be an important consideration for a clinical setting. In contrast there can be no debate that the inclusion of antibiotics in medium for the preparation of sperm is a prerequisite.

Protein/Macromolecules

Historically, the most commonly used protein source in human IVF and embryo culture was patient's serum, added to the culture medium at a concentration of 5% to 20%. In some programs, fetal cord serum was used in preference. The use of serum in embryo culture media has several inherent drawbacks: the considerable expense and time required for its collection and processing (and screening of the fetal cord serum), the risk of infection to the laboratory staff, as well as the added stress to the patient. Serum contains many components which are poorly characterized. Furthermore, proteins in serum have macromolecules attached, such as hormones, vitamins and fatty acids, as well as chelated metal ions and pyrogens (184,185). As the concentration

of such macromolecules and other serum components varies between patients and even within the menstrual cycle, it makes any comparison between batches of medium which contain serum almost impossible. Furthermore, serum from several groups of patients such as those with endometriosis, PCO or unexplained infertility appears to be embryo-toxic (186–190). There are several reasons for the elimination of serum from mammalian embryo culture systems. From a physiological perspective the mammalian embryo is never exposed to serum in vivo. The fluids of the female reproductive tract are not simple serum transudates (191), but rather specialized environments for the development of the embryo (30). Serum can best be considered a pathological fluid formed by the action of platelets. More disturbing however, is the growing evidence that serum is detrimental to the developing mammalian preimplantation embryo in culture. Studies on the embryos of mice, sheep and cattle have demonstrated that serum in the culture medium induces morphological, metabolic, genetic and ultrastructural changes in blastocysts cultured from the zygote stage. The trophectoderm of such blastocysts develops a vesicular appearance due to the sequestering of lipid in the blastomeres (37,102,128,192,193).

Furthermore, when ruminant pronucleate embryos were cultured to the blastocyst stage in the presence of serum they possessed mitochondria with abnormal folding of the cristae, possibly associated with reduced oxidative capacity (128,192). Such blastocysts exhibited elevated levels of lactate production, plausibly associated with mitochondrial damage and impaired oxidative capacity (102). Finally, the inclusion of serum in the culture medium is associated with the birth of abnormally large lambs after the transfer of blastocysts to recipient ewes (128,194). Such data is of great concern and the mechanism(s) by which serum imparts such detrimental effects is the focus of much research. The over expression of certain growth factor genes in this phenomenon is a plausible mechanism (195–197). For example, fetal overgrowth in the sheep following embryo culture in the presence of serum has been associated with a decreased expression of M6P/IGF-IIR through loss of methylation (196). M6P/IGF-IIR has a role in fetal organogenesis. Interestingly, this locus although imprinted in mice, sheep and cows, is not imprinted in the human (198). Subsequently, this specific absence of imprinting may mean that the human embryo is less susceptible to epigenetic disturbances. This may therefore explain why Menezo and colleagues (199) have not reported any adverse effects in children following blastocyst co-culture in the presence of serum, but that studies on mice, sheep and cattle have all revealed long term effects on embryos cultured in the presence of serum.

So why was serum included in human embryo culture media? Undoubtedly the main reason for the inclusion of serum in media used in human IVF is the limited ability of simple salt solutions and tissue culture media to support embryo development in the absence of serum. In a suboptimal medium serum can act as a chelator and a buffer to minimize pH fluctuations when

medium is outside of a CO_2 environment. It may serve to supplement simplistic media with known regulators of embryo development such as amino acids, whereas when added to more complex tissue culture media such as Ham's F-10, it may help by binding the embryo toxic transitional metals present. However, with the development of more physiological embryo culture media designed to fulfill the changing requirements of the embryo, and the inclusion of appropriate chelators, the requirement for serum in embryo culture has been eliminated (37,38,40,46,200). Serum should now reside only in the annals of embryo culture, and certainly not in the media.

Protein can be added to culture media in the form of serum albumin. The addition of a macromolecule such as serum albumin prevents gamete and embryos from becoming "sticky" whereby their surface charges make them stick to both glass and plastic. Macromolecules, therefore, facilitate gamete and embryo manipulation. Furthermore, albumin can negate the effects of toxins (201). Both human serum albumin (HSA) (46,202–204) and bovine serum albumin (BSA) (205) have been used successfully in the culture of human embryos. The use of HSA requires adequate screening for HIV, hepatitis etc., while the use of animal products in human ART is no longer acceptable. More recently, several commercially available serum products have been used to great success in replacing serum in human embryo culture systems. These range from therapeutic albumin solutions (202,204,206) to globulin enriched albumin solutions such as Plasmanate (207), Plasmatein (208) and Synthetic Serum Substitute (SSS) (209–211). Of the latter products, SSS appears to be the most effective containing 84% HSA and 16% α- and β-globulins with less than 1% γ-globulin. It has been proposed that the glycoprotein components of serum (α- and β-globulins) have a role in supporting embryo development in culture. Glycoproteins, which possess numerous hydroxyl groups, may confer benefit to the embryo by altering the solvent properties of the medium, making it more akin to the tubal environment (208,212). However, there have been no prospective randomized trials using such supplements.

Although serum albumin is a relatively pure fraction, it is still contaminated with fatty acids and other small molecules (213). The latter includes an embryotrophic factor, which stimulates cleavage and growth in rabbit morulae and blastocysts. This factor has been determined to be citrate (214). Not only are there significant differences between sources of serum albumin (215,216), but also between batches from the same source (215,217). Furthermore, some HSA preparations contain the preservative sodium caprylate, which binds to the hydrophobic domains of the proteins and therefore cannot be removed by dialysis (Pool TB. Personal communication, 1998). The effects of such a preservative on embryo development have yet to be determined. Therefore when using serum albumin or any albumin preparation, it is essential for each batch to be screened for its ability to adequately support embryo development in the mouse prior to

clinical use. Importantly, recombinant human serum albumin has recently become available, which eliminates the problems inherent with using blood derived products, and certainly eliminates variability between lots. The use of recombinant human albumin has been validated in a prospective randomized trial and has been found to be equally as effective as human serum albumin in supporting IVF, embryo development in vitro, and subsequent pregnancies (218,219). Furthermore, the inclusion of recombinant human albumin appears to confer increased cryotolerance to those embryos cultured in its presence (11).

Finally, there is much interest in developing alternative macromolecules to serum albumin, thereby facilitating the formulation of more defined culture media. The synthetic polymer polyvinyl alcohol (PVA) has been used extensively by Bavister's laboratory (220), although its effects on post-implantation development have yet to be fully elucidated in prospective trials. A major component of oviduct and uterine fluids are glycosaminoglycans and proteoglycans. Similar to glycoproteins, glycosaminoglycans and proteoglycans have the capacity to attract cations such as sodium due to their high density of negative charges. Therefore these molecules will also alter the solvent properties of the medium. The glycosaminoglycan hyaluronate can substitute for albumin when added to the culture medium and increase the cryotolerance of embryos (219,221,222). Interestingly, albumin and hyaluronate act in synergy to further increase mouse blastocyst development in culture. The addition of hyaluronate to the culture medium has also been shown to increase blastocyst development in porcine embryos (223). Of greatest significance however is the finding that the addition of hyaluronate to embryo culture medium significantly increases mouse blastocyst implantation and fetal development after transfer (221). Interestingly, this increase in viability was found to be due to the presence of hyaluronate in the medium used for transfer (Fig. 4) (221). As the human endometrium and embryo expresses the receptor for hyaluronate (224), it is plausible that hyaluronate is involved in the initial phases of blastocyst attachment to the endometrium. Therefore studies on the role of such glycosaminoglycans in human embryo development and transfer are warranted.

Hormones and Growth Factors

Although the mouse blastocyst is capable of metabolizing exogenous steroid hormones (225), there is limited data on the direct action of hormones on the early embryo (226–229). Certainly, estradiol appears to have a direct negative impact on mouse embryo development (230,231). However, prolactin at a concentration of 300 ng/mL, has been shown to improve the rate of blastocyst formation from cultured 2-cell mouse embryos (232). Available evidence indicates that the effects of maternal hormones on the developing embryo are mediated through the cells of the reproductive tract (233).

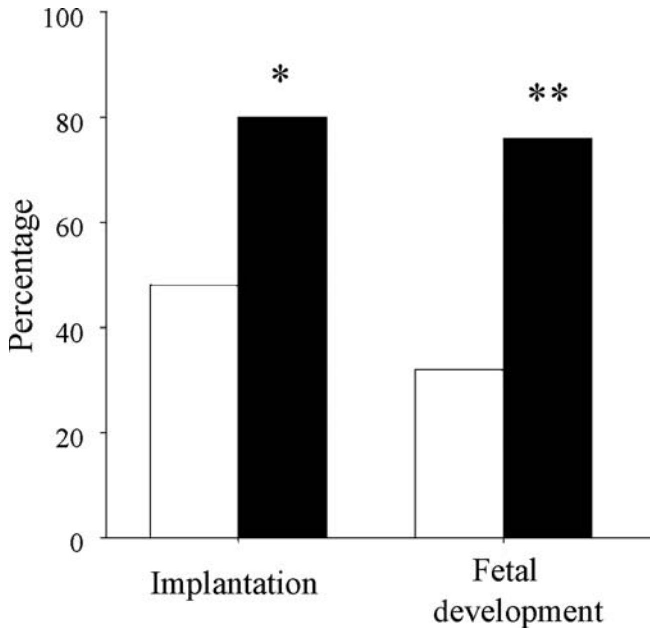

Figure 4 Effect of hyaluronate in the transfer medium on subsequent viability of cultured mouse blastocysts. Mouse zygotes were cultured to the blastocyst stage in sequential media DM2/DM3 without protein or macromolecule supplement. Blastocysts were placed in either medium DM3 or DM3 containing hyaluronate and transferred to pseudopregnant recipients and subsequent viability was assessed. *Open bars* represent medium DM3 without protein or macromolecule. *Solid bars* represent medium DM3 supplemented with hyaluronate. *Note*: *Significantly different to medium DM3 ($P < 0.05$). **Significantly different to medium DM3 ($P < 0.01$). *Source*: From Ref. 221.

The role of growth factors in the development of the preimplantation mammalian embryo has been the subject of intensive research in recent years resulting in a wealth of literature. Studies on the embryos from several mammalian species have identified the transcripts for the ligand and/or receptor for the following: insulin, IGF-I, IGF-II, EGF, TGF-α, TGF-β, PDGF-A, FGF-4, LIF. These growth factors have been shown to stimulate either cleavage, amino acid transport, protein synthesis, blastocoel formation, or ICM development. Several detailed reviews on the action of specific growth factors have now been published (234–239). In the human there is growing evidence that growth factors are present within the female tract and embryo. Martin et al. (240) demonstrated that adding heparin-binding epidermal growth factor to the culture medium for human embryos significantly increased both blastocyst development and subsequent hatching.

Similarly, the addition of IGF-I stimulated blastocyst formation and increased development of the ICM (241). Therefore, growth factors are potential regulators of human embryo development (242–245). However, it is possible that synergies may exist between two or more growth factors and that simply adding an individual growth factor may not result in a response by the embryo. Furthermore, many growth factors exhibit pleiotropic properties such that cells can be directed down certain pathways of differentiation, even improper pathways, by exposure to inappropriate growth factors.

Platelet-activating factor (PAF) has been implicated as one of the earliest embryonic signals produced and its appearance, or otherwise, in culture medium associated with viability. Furthermore, it has also been shown that the addition of exogenous PAF is associated with an increase in pregnancy rate (246).

However, as most human embryos are currently transferred around the 2- to 8-cell stage, it is likely that expression of almost all growth factor receptors occurs after the embryo is transferred to the female tract. Due to the high costs of growth factors for media supplementation and the lack of data on their role in human embryo development, it is considered prudent that they are not included in media formulations for clinical IVF. With the advent of culture systems for prolonged embryo culture, the inclusion of growth factors in culture media will have to be re-evaluated, though extreme care and extensive animal studies are required, especially at the molecular level.

Buffer System

Most embryo media utilize a bicarbonate/CO_2 buffer system to maintain a physiological pH of between 7.2 and 7.4 in the medium. The inclusion of sodium bicarbonate in the medium requires the use of a CO_2 incubator to maintain a 5–7% CO_2 atmosphere. An advantage of the bicarbonate/CO_2 system is that it is the physiological buffering system in fluid surrounding mammalian cells. A major drawback, however, is the rapid pH increase that occurs when medium is exposed to air, resulting in impaired embryo development or even cellular necrosis if prolonged. A practical solution to this problem is the use of an oil overlay to reduce gas exchange when culture dishes are taken out of the incubator, e.g., for embryo scoring. This approach is not feasible however, for oocyte collection or prolonged embryo manipulation. A possible alternative, therefore, is the use of phosphate-buffered medium, which does not require a CO_2 environment to maintain its pH in air. Unfortunately, phosphate-buffered media appear to be detrimental to embryo development in vitro (247–249). The detrimental effect of phosphate on embryo development is exacerbated in a simple medium, such as phosphate-buffered saline (PBS), i.e., the reduced rates of development

associated with phosphate would be at its greatest in a medium such as PBS. Furthermore, during cryopreservation procedures, PBS has limited buffering capacity, therefore, rendering the embryos susceptible to pH stress. Therefore, the use of PBS for embryo collection, manipulation or cryopreservation should be avoided. An alternative to a phosphate buffer system is N′-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), with a pK_a of 7.55 at 20°C (250). HEPES has been used successfully as a buffer in media for human oocyte collection and embryo handling. In such media, it is usual to replace 20 mM bicarbonate with HEPES, leaving 5 mM bicarbonate (251). The rationale for this is that embryos require bicarbonate for development (78,249). At lower concentrations of HEPES (<20 mM), the bicarbonate competes with the HEPES, which subsequently loses its buffering capacity as the bicarbonate dissociates and consequently increases the pH (252). More recently another zwitterionic buffer 4-Morpholinepropanesulfonic acid (MOPS) (250) has been used successfully for embryo handling and manipulation. A potential advantage of MOPS over HEPES is that its buffering capacity is less temperature dependent than HEPES.

CULTURE SYSTEM

Optimization of embryo development in vitro is not only dependent upon the composition of the culture medium or media used, but is also affected by physical parameters, such as the incubation environment and gas phase.

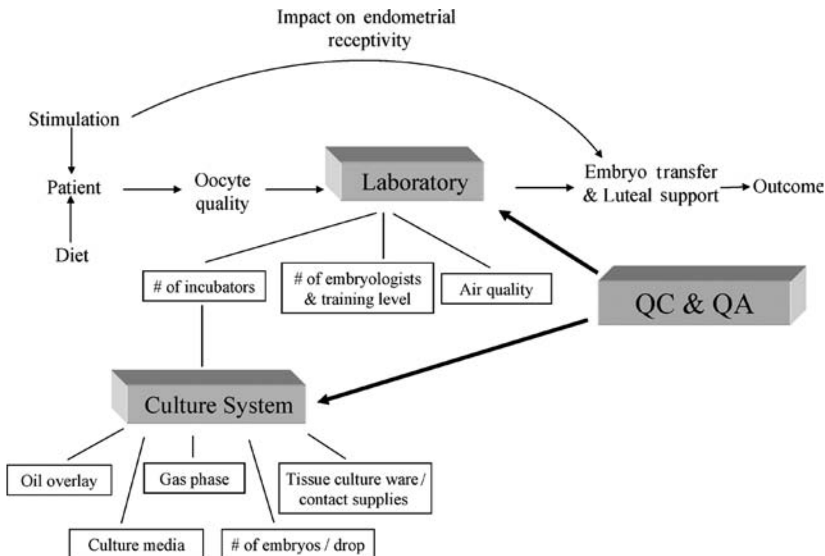

Figure 5 (*Caption on facing page*)

Therefore, it is important to consider the "culture environment" when attempting to improve embryo culture systems (Fig. 5).

Oxygen

The concentration of oxygen in the lumen of the rabbit oviduct is reported to be 2–6% (259,260). It was subsequently determined that the oxygen concentration in the oviduct of hamster, rabbit and rhesus monkey was similar at 8% (261). However, the oxygen concentration in the uterus was significantly lower than in the oviduct ranging from 5% in the hamster and rabbit to 1.5% in the rhesus monkey (261,262).

Studies on several types of mammalian embryo have demonstrated that culture at a reduced oxygen concentration results in enhanced development in vitro (263). In the mouse, culture at oxygen concentrations as low as

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Figure 5 (*figure on facing page*) A holistic analysis of human IVF. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts oocyte quality (hence embryo physiology and viability), but can also affect subsequent endometrial receptivity. Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo. The dietary status of patients attending IVF is typically not considered as a compounding variable, but growing data would indicate otherwise. In this schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down into its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e., culture media), that one is going to mimic the results of a given laboratory or clinic. A major determinant of the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pre-tested with a relevant bioassay (for example, mouse embryo assay) is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF come suitably tested. Therefore, it is essential to assume that everything entering the IVF laboratory without a suitable pretest is embryo toxic until proven otherwise. In our program, the 1-cell mouse embryo assay (MEA) is employed to prescreen every lot of tissue culture ware that enters the program, i.e., plastics that are approved for tissue culture. Around 25% of all such materials fail the 1-cell MEA (in a simple medium lacking protein after the first 24 hours). Therefore, if one does not perform QC to this level, one in four of all contact supplies used clinically could compromise embryo development. In reality, many programs cannot allocate the resources required for this level of QC; and when embryo quality is compromised in the laboratory, it is the media that are held responsible, when in fact the tissue culture ware is more often the culprit. *Abbreviations*: IVF, In vitro fertilization; MEA, mouse embryo assay; QC, quality control; QA, quality assurance. *Source*: From Refs. 131, 140, 253–258.

1% were sufficient to support embryo development to the blastocyst stage (264). Furthermore, several other studies have shown that a reduced oxygen concentration, between 5% and 8%, enhances development to the blastocyst stage in the mouse (105,164,264,265). Similarly, studies on the rabbit (266) and on domestic animal species such as sheep (263), goats (215), and cows (263) have also demonstrated that an oxygen concentration of 7% results in increased development in vitro compared to 20% oxygen. Furthermore, it has been observed that human blastocysts cultured in a low oxygen environment (5%) have significantly more cells than those cultured in a high oxygen environment (20%) (Gardner DK. Unpublished Data. 1998). Even a transient exposure for one hour to 20% oxygen reduced mouse embryo development in vitro (263). It has subsequently been determined that equilibrating culture dishes at 20% oxygen for five hours prior to culture in 7% oxygen decreases mouse zygote development to the blastocyst stage and resultant blastocyst cell numbers. This inhibition can be attributed to the fact that it takes more than five hours for the oxygen concentration to fall to embryo-safe levels (105).

Interestingly however, human and mouse embryos can grow at elevated oxygen concentrations and this has lead to some confusion regarding the optimal concentration for embryo culture. The physiology of the reproductive tract and the beneficial effects of using a reduced oxygen concentration as determined in controlled studies indicate that to employ low oxygen concentrations appears prudent.

Carbon dioxide

Carbon dioxide is not only required to maintain the pH of bicarbonate buffered medium, but is readily incorporated into protein and nucleic acids by the mouse embryo at all stages prior to implantation (267). Culture systems for the preimplantation mammalian embryo routinely employ a carbon dioxide concentration of 5% coupled with a bicarbonate concentration of around 25 mM. However, analysis of the uterine environment in the guinea pig on day 4 of pregnancy determined the carbon dioxide concentration to be 10% (268). Studies on the hamster 8-cell embryo showed that development to the blastocyst stage is increased by a carbon dioxide concentration of 10% (53,269). Similarly, rabbit zygote development to the hatching blastocyst stage is increased in a carbon dioxide concentration of 10% compared to 5% (270,271). It has been proposed that the beneficial effects of a high carbon dioxide concentration is due to a decrease in the pH of the medium as the beneficial effects of increased carbon dioxide could be replaced with a weak acid (269). The pH of media containing 25 mM bicarbonate that is equilibrated in a CO_2 environment can be calculated using the Henderson–Hasselbach equation. Interestingly, at 5% CO_2 the pH of medium containing 25 mM bicarbonate is 7.45, while a CO_2 concentration

of 6% is required to maintain the external pH of the medium at around 7.4. The optimal concentration of carbon dioxide for human embryo development has yet to be determined; however the CO_2 concentration is related to pH of the medium which also needs to be considered. Typically, Fyrite has been employed to quantitate CO_2 within the chamber used. However, the accuracy of such a procedure is rather low. Fortunately, there are now available new hand-held infra red (IR) CO_2 analysis meters with very high accuracy (to 0.1%) (e.g., Vaisala, Helsinki, Finland).

pH

The pH of fluid collected from the reproductive tract of rhesus monkey was reported to alter in parallel with changes in the bicarbonate concentration increasing from 7.1 to 7.3 during the follicular phase of the estrous cycle to 7.5–8.0 at the time of ovulation and during luteal phase (272). Dale et al. (273) recently reported that the pH of the uterine fluid is lower than that of the oviduct. External pH of culture media formulated for preimplantation embryos is commonly between 7.3 and 7.4 (depending on the CO_2 concentration used). Studies on the mouse embryo determined that development from the 2-cell stage to the blastocyst stage could occur in media with a pH range from 5.9 to 7.8 (65). Similarly, hamster 8-cell embryos could develop to the blastocyst stage in medium with an external pH range from 6.4 to 7.4 (269). In contrast however, it has recently been shown that a transient exposure of zygotes and 2-cell mouse embryos to medium with elevated pH significantly reduced subsequent development to the blastocyst stage (274). Although these studies demonstrated that some embryos could still develop to the blastocyst stage in a wide range of external pH's, the subsequent viability of these embryos following transfer is unknown. Subsequently, there has been considerable work in the area of intracellular pH (pHi) and its role in regulating embryo development. It has been established that even relatively small fluctuations in pHi can significantly retard subsequent developmental competence. Fluctuations in either the acidic (84,275) or the alkaline (276) range can drastically reduce development. Even more significantly, it has been determined that mammalian oocytes and embryos for around six hours following fertilization lack any functional transport systems to regulate pHi in either the acid (275) or the alkaline (275,277) ranges. Therefore, care should be taken to avoid fluctuations in the pH of media during embryo manipulation and culture. This is especially relevant for oocytes that are stripped of their cumulus before an ICSI procedure. Immediately following the denudation procedure these oocytes and embryos cannot regulate their ionic homeostasis. Amino acids are also known to increase the intrinsic buffering capacity of the embryo and reduce fluctuations in intracellular pH of the embryo. Therefore, as discussed earlier, addition of amino acids to the culture or handling medium

Table 7 Preparation of Color Standards for pH of Media

pH at 18°C	Solution A (mL)	Solution B (mL)
6.6	62.7	37.3
6.8	50.8	49.2
7.0	39.2	60.8
7.2	28.5	71.5
7.4	19.6	80.4
7.6	13.2	86.8

Note: Stock A: 9.08 g KH_2PO_4 (0.067 M), 10 mg phenol red in 1 L of water. Stock B: 9.46 g Na_2HPO_4 (0.067 M), 10 mg phenol red in 1 L of water. Measure the pH with meter, and adjust pH as required, i.e., add solution A to lower the pH (make more acidic), add solution B to increase the pH (make more alkaline). The pH standards should be filter-sterilized and can then be kept for up to six months.

can also help to reduce pHi fluctuations (31). It is also important to consider the relationship between the pH of the culture medium (pHo) and the pHi of the embryo; over a pHo range of 7.0–7.6, the pHi of the mouse zygote remained at 7.17. Rather than pHo affecting pHi directly, the presence of weak acids or bases in the culture medium dramatically affects pHi (84).

The pH of a CO_2/bicarbonate buffered medium is not easy to quantitate. A pH electrode can be used, but one must be quick and the same technician must take all readings to ensure consistency. Solid state probes are now available with a higher degree of accuracy. An alternative approach is to take samples of media and measure the pH with a blood-gas analyzer. A simple and reliable method of checking the pH of a medium is to use color standards. The color standards use 0.067 M solutions of potassium phosphate and sodium phosphate. These solutions are then added together in varying quantities to produce solutions of the required pH. The preparation of such standards is shown in Table 7. However, this approach requires the medium to contain phenol red.

Temperature

Temperature fluctuations at the early cleavage stages have been demonstrated to decrease the subsequent development potential of embryos in vitro. Exposure of mouse zygotes to room temperature for just five minutes reduced cleavage rates. Increasing the exposure time to 10 and 15 minutes further decreased cleavage rates and reduced development to the blastocyst stage such that blastocyst development was half of the control after 15 minutes at room temperature (274). Exposure of rabbit cleavage stage embryos to room temperature for three hours also decreased cleavage rates as assessed by thymidine incorporation and development to morulae and blastocyst stages (278). Exposure of human oocytes to room

temperature has been reported to induce damage to the meiotic spindle (279). It would therefore seem advisable to maintain a constant temperature of 37°C when handling human oocytes and embryos.

Incubator/Incubation Chamber

As discussed above, for optimal development of human embryos in vitro it is important to maintain both the pH of the medium and temperature. The choice and use of incubators is therefore paramount for the success of an IVF program. Several studies have determined that embryo development in vitro is increased by restricting the opening of an incubator (105,274). Furthermore, the use of IR sensor incubators which restore CO_2 levels within two to three minutes helps to alleviate the detrimental effect of repeated opening of the incubator (274). Alternatively, the use of modular incubator chambers (MICs) inside the incubator enables the gas phase that embryos are cultured in to remain constant. Mouse embryo development in MIC's at the same gas phase as the incubator (5% CO_2 in air) was significantly increased compared to development in the main chamber of the incubator (105). The use of incubators that have multiple chambers within the single incubator chamber can also reduce fluctuations in temperature and CO_2 levels induced by frequent door openings.

Light

Several studies have investigated the effect of exposure to visible light on the development of mammalian preimplantation embryos in vitro. Collection and culture of embryos from the hamster (280), and rabbit (278,281) under low illumination increased development and cleavage in vitro. Exposure of hamster oocytes for one hour to visible light prior to insemination disrupted the completion of meiosis and fertilization (282). In the human, implementation of an oocyte collection system employing low light at low oxygen concentration resulted in significantly increased rates of blastocyst formation of spare embryos and increased pregnancy and live birth weights when embryos were transferred on days 2–4 of culture (283). Bedford and Dobrenis (284) reported that a 20–30 minute exposure of rabbit oocytes to light did not affect subsequent fertilization in vivo or resultant pregnancy rates. However, these oocytes were immediately transferred to the reproductive tract of a recipient and not maintained in culture. Therefore, it would seem prudent to perform all oocyte and embryo collections and manipulations under low illumination.

Incubation Volume/Embryo Grouping

Within the lumen of the female reproductive tract, the developing embryo is exposed to microliter volumes of fluid (191). In contrast, the embryo grown

in vitro is subject to relatively large volumes of medium up to 1 mL. Consequently, any autocrine factor(s) produced by the developing embryo will be diluted and may therefore become ineffectual. It has been demonstrated in the mouse that cleavage rate and blastocyst formation increase when embryos are grown in groups (up to 10) or reduced volumes (around 20 μL) (57,285–287). Of greatest significance is the observation that decreasing the incubation volume significantly increases embryo viability (286) due to an increase in ICM development (288,289). Similar results have been obtained with sheep (102) and cow embryos (289). It is therefore apparent that the preimplantation mammalian embryo produces a factor(s) capable of stimulating development of both itself and surrounding embryos (Fig. 6) (290). Once the identity of the factor(s) responsible for this stimulation of development has been determined, it can be added to the culture medium to optimize the embryo's response.

In order to culture in such reduced volumes (20–50 μl), an oil overlay is required. Although the use of an oil overlay is time-consuming, it prevents the evaporation of media, thereby reducing the harmful effects of increases in osmolality, and reduces changes in pH caused by a loss of CO_2 from the medium when culture dishes are taken out of the incubator for embryo examination. Furthermore, it has been proposed that an overlay of mineral oil can actually act as a trap for potential embryo toxins (291). If oil is to be used then paraffin or light mineral oils are recommended. Unfortunately paraffin oil has the disadvantage of being highly labile and therefore has a relatively short shelf-life. However, in a busy laboratory the oil is used before it has a chance to become toxic. It is advisable to purchase paraffin oil in small bottles (100–500 mL) to ensure rapid use once it is opened. It is essential that each bottle of oil purchased is tested using the mouse bioassay prior to clinical use. The use of silicone oil has also been advocated for embryo culture, however it appears that embryo-toxic zinc can be a contaminant of this oil (292).

The washing of oil with medium is commonly recommended. However, the washing of oil has no effect on embryo development provided the oil has passed the quality control (mouse embryo bioassay; see below). Therefore, an oil which was borderline in the quality control can be made to support higher rates of embryo development after washing. However, such bottles of oil should not normally be accepted in the first place.

WHAT STAGE SHOULD THE HUMAN EMBRYO BE TRANSFERRED?

It has been an accepted global practice to transfer embryos on day 2 (around the 4-cell stage) or on day 3 (around the 8-cell stage) of development. However, such cleavage stage embryos reside in the fallopian tube and not in the

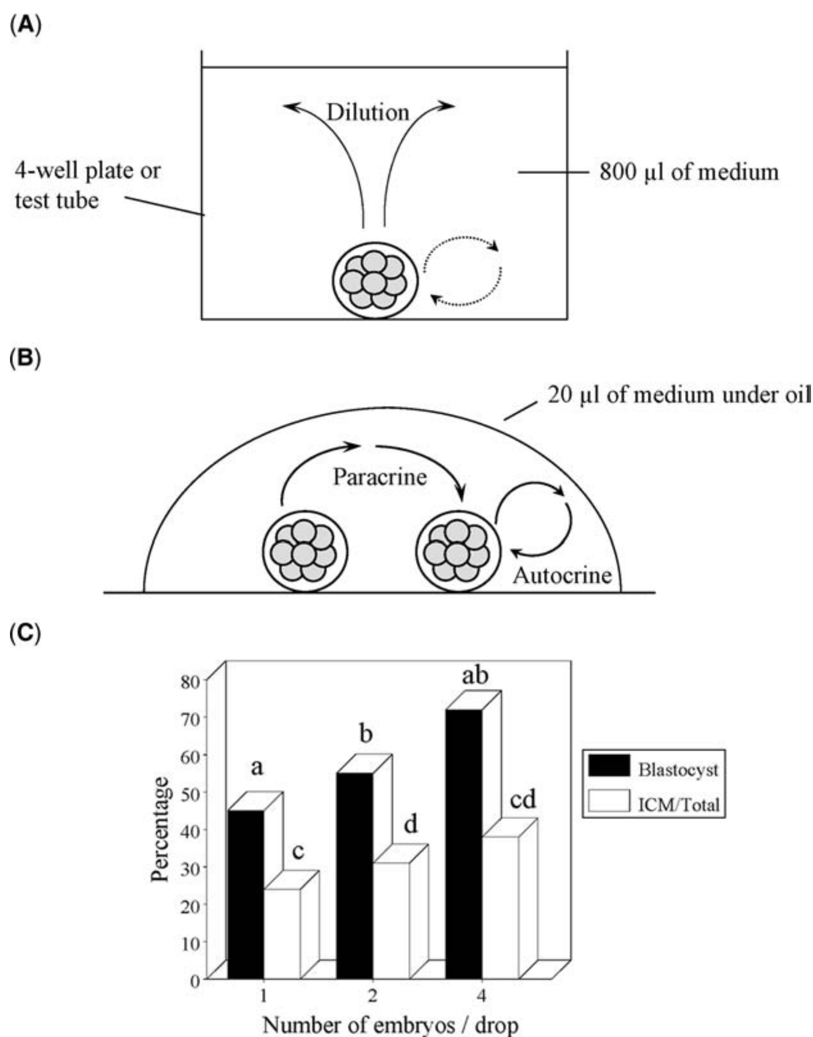


Figure 6 Effect of incubation volume and embryo grouping on embryo development and differentiation. (**A**) A single embryo cultured in a 4-well plate or test tube, any factor produced by the embryo will become ineffectual as a result of dilution. (**B**) Culture of embryos in reduced volumes and/or groups increases the effective concentration of embryo-derived factors, facilitating their action in either a paracrine or autocrine manner. (**C**) Effect of embryo grouping on bovine blastocyst development and differentiation. Bovine embryos were cultured either individually or in groups of two or four in 50 µl drops of medium. Like pairs are significantly different ($P < 0.05$). *Abbreviation*: ICM, inner cell mass. *Source*: From Refs. 289 and 290.

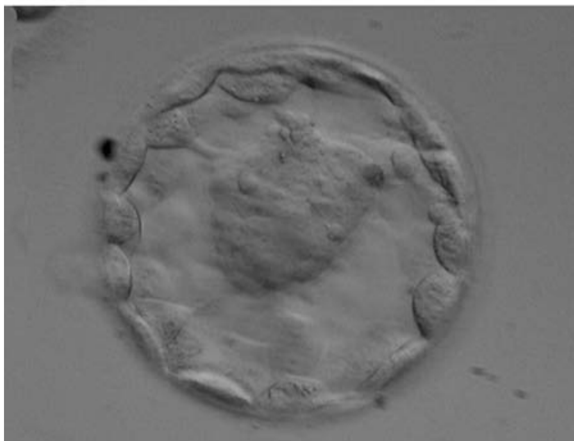
uterus (293). The significance of this observation is that in other mammalian species the transfer of cleavage stage embryos to the uterus results in lower pregnancy rates than are attained by the transfer of post compaction or blastocyst stage embryos (8). With the development of more effective culture media, it has become possible to culture human embryos to the blastocyst stage as a matter of routine (294,295).

The potential advantages of blastocyst culture and transfer in human IVF include:

1. The synchronization of the embryo with the female tract (296), leading to increased implantation rates (297), therefore reducing the need for multiple embryo transfers.
2. The ability to assess embryo development and viability over extended culture. This can be achieved by both the identification of those embryos with little developmental potential, as manifest by slow development or degeneration in culture, and by the introduction of non-invasive tests of developmental potential to select the most viable embryos from within a cohort for transfer.
3. Minimizing the exposure of the embryo to a hyperstimulated uterine environment.
4. Culture for an extra 2–3 days increases the time available between cleavage stage embryo biopsy and the time of transfer. This is of particular importance where the biopsied material has to be sent to a separate locale for analysis.
5. Assessment of true embryo viability, i.e., assessing the embryo post genome activation (298).
6. Reduced uterine contractions on day 5 of embryo development, minimizing the chance that an embryo will be expelled from the uterus (299).
7. Increased ability to undergo cryopreservation (300–302).
8. The generation of blastocysts will facilitate the introduction of trophectoderm biopsy for the screening of genetic diseases. Trophectoderm biopsy represents the earliest form of genetic diagnosis of non-embryonic material.
9. Reduced pregnancy loss (303).

From such a list, it would appear that blastocyst transfer on day 5 offers several advantages over the convention of transferring early embryos on days 1 to 3 of development. However, many clinics around the world have yet to adopt extended culture and blastocyst transfer. In a review on blastocyst vs. cleavage stage transfer (304), it was noted that in most all papers published on blastocyst transfer, very little information regarding the culture system, save the medium type, was ever reported. This in turn makes comparing various studies rather problematic. An analysis of 16 prospective randomized trials revealed that 8 studies found a positive outcome

(A)



(B)

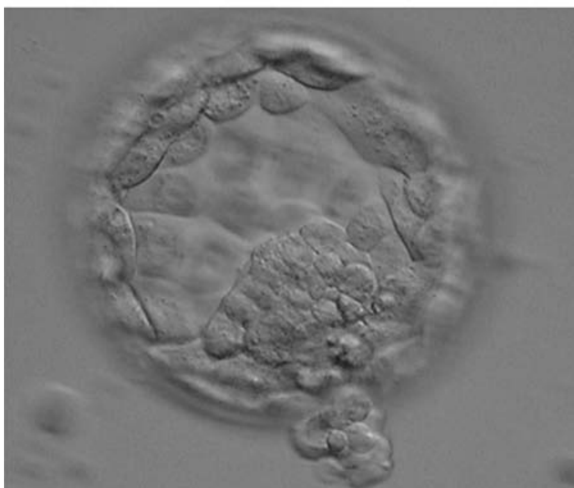


Figure 7 Photomicrographs of a human blastocyst. (**A**) Human blastocyst on the morning of day 5 after culture in medium G 1 for 48 hours from the pronucleate stage, followed by culture in medium G 2 for 48 hours from the 8-cell stage. Note the thinning of the zona pellucida. A dense group of cells, the inner cell mass, can be seen in the middle of the blastocyst. The embryo would be scored as 4AA. (**B**) Human blastocyst cultured under the same conditions as the embryo in (**A**). This blastocyst has commenced hatching and would be scored as 5AA.

when blastocysts were transferred compared to transfers on day 2 or day 3, with only one finding a negative outcome. The remaining seven demonstrated equivalency between days of transfer. However, more and more

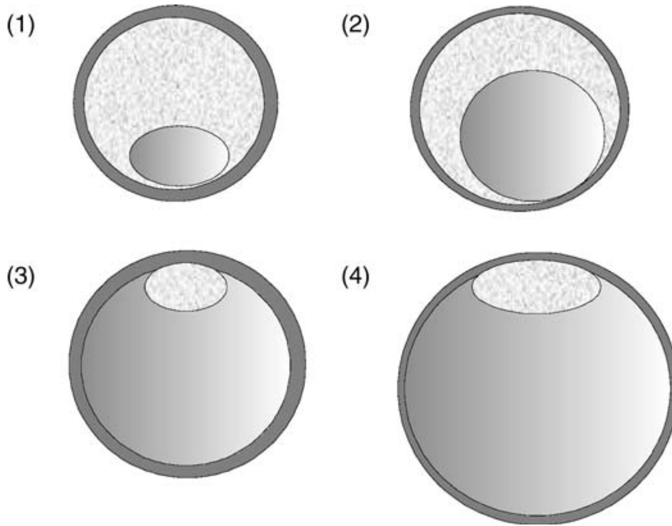


Figure 8 Scoring system for human blastocysts. Initially blastocysts are given a numerical score from 1 to 6 based upon their degree of expansion and hatching status: (1) early blastocyst; the blastocoel being less than half the volume of the embryo; (2) blastocyst; the blastocoel being greater than or equal to half of the volume of the embryo; (3) full blastocyst; the blastocoel completely fills the embryo; (4) expanded blastocyst; the blastocoel volume is now larger than that of the early embryo and the zona is thinning; (5) hatching blastocyst; the trophectoderm has started to herniate through the zona; (6) hatched blastocyst; the blastocyst has completely escaped from the zona. The initial phase of the assessment can be performed on a dissection microscope. The second step in scoring the blastocysts should be performed on an inverted microscope. For blastocysts graded as 3 to 6 (i.e., full blastocysts onwards) the development of the inner cell mass (ICM) and trophectoderm can then be assessed. ICM grading: A—tightly packed, many cells; B—loosely grouped, several cells; C—very few cells. Trophectoderm grading: A—many cells forming a tightly knit epithelium; B—few cells; C—very few cells forming a loose epithelium. *Source*: From Ref. 307.

reports are now leaning to blastocyst transfer, especially with regard to single embryo transfer (303,305,306). Furthermore, it appears that cryopreservation of human embryos is most effective at the blastocyst stage (300,302). Consequently, extended culture, facilitated by the culture systems outlined in this chapter, may represent a way to increase the overall efficiency of human IVF.

Figure 7 shows the morphology of human blastocysts following culture in sequential media, together with an alpha-numeric scoring system (Fig. 8). It is evident that blastocyst score is correlated with subsequent implantation potential (307), with implantation rates of $>60\%$ being attainable.

SUMMARY

In contrast to the female reproductive tract, most conventional embryo culture systems are static, employing a single medium for the entire preimplantation period. In vivo, the embryo is exposed to a constantly changing environment as it passes along the oviduct to the uterus. Concomitantly, the embryo itself exhibits a changing nutrient preference (70–72,75,76), reflecting the changes in physiology and energy metabolism which occur between fertilization and the blastocyst (7,34,80,88,98). Furthermore, there is the added problem of in vitro induced artifacts, such as the build up of potential toxins in the medium over time, if it is not renewed.

It is therefore argued that optimal conditions for IVF and the subsequent development of the mammalian zygote to the blastocyst stage in culture requires more than one culture medium, rather than letting the embryo adapt to a static environment as its requirements change during development and differentiation.

The role of the cumulus in early embryo development has all but been ignored. However, it is now evident that the cumulus cells provide many important functions not just for the oocyte but also for the very early embryo undergoing fertilization, i.e., pronuclei formation, polar body extrusions, organelle redistribution, and cytoskeletal rearrangements. The cumulus not only provides a microenvironment that consists of low levels of glucose and high lactate concentrations, but also provides homeostatic regulation for the oocyte and early embryo. Furthermore, analysis of cumulus physiology and gene expression may assist in the diagnosis of a competent oocyte (308,309). In procedures such as ICSI, where the cumulus is stripped from the oocyte, the oocyte lacks any system to regulate the intracellular environment. Therefore, extra care must be taken in the handling of the oocyte before and after ICSI, and all media must contain amino acids in order to prevent homeostatic stress (105).

Finally, optimal culture conditions for the mammalian embryo depend on more than just media composition. Figure 5 attempts to highlight the interconnectedness of all components of an IVF cycle. It is hoped that this model will assist not only in improving the performance of a given laboratory, but will help in the troubleshooting.

EMBRYO CULTURE PROTOCOL

The protocol described below is based on the assumption that the media in question will be renewed after 48 hours. As discussed above, this is paramount whether one is using a biphasic or non-sequential system. It has been validated using the media and consumables listed. Any change to the protocol, whether it be a different source of oil or media needs to be validated carefully. When using the sequential media G1 and G2 (131),

embryos should be cultured in a gas phase of 5% O_2 and 6% CO_2. Remember, human embryos will grow at 20% O_2, but development is superior at lower oxygen tensions.

Pronucleate Stage Embryos to Day 3 Culture

All manipulations of oocytes and embryos should be performed using a pulled Pasteur pipette, glass capillary or a displacement pipette. It is important to use a pipette with the appropriate size tip. For example, once the cumulus is removed (day 1 to 3), a bore of around 175–200 μM is required. Using the appropriate size tip minimizes the volumes of culture medium moved with each embryo, which typically should be less than a microliter. Such volume manipulation is a pre-requisite for successful culture.

Around 4 PM on the day of oocyte retrieval, label 60 mm Falcon Primaria dishes with the patient's name. Using a single-wrapped tip, first rinse the tip, then place 6×25 μl drops of G1 into the plate. Four drops should be at the 3, 6, 9, and 12 o′ clock positions (for embryo culture), the fifth and sixth drops should be in the middle of the dish (wash drops). Immediately cover drops with 9 mL of tested oil (such as Ovoil, Vitrolife). Prepare no more than 2 plates at one time. Using a new tip for each drop, first rinse the tip and then add a further 25 μl of medium to each original drop. Place the dish in the incubator at 5% O_2 and 6% CO_2. Gently remove the lid of the dish and set at an angle on the side of the plate. Dishes must gas in the incubator for a minimum of four hours (this is the minimal measured time for the media to reach correct pH under oil). For each patient, set up a wash dish at the same time as the culture dishes. Place 1 mL of medium G1 into the center of an organ well dish, place 2 mL of medium into the outer well, and then place the dish in the incubator. If working outside an isolette, use a MOPS or HEPES buffered medium with amino acids. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage.

Following removal of the cumulus cells, embryos are transferred to the organ well dish and washed in the center well drop of medium in the culture dish. Washing involves picking up the embryo 2–3 times and moving it around within the well. Embryos should then be washed in the two center drops in the culture dish and up to 5 embryos placed in each drop of G1. This will result in no more than 20 embryos per dish. Return the dish to the incubator immediately. It is advisable to culture embryos in groups of at least 2. Therefore for example, for a patient with 6 embryos, it is best to culture in 2 groups of 3 and not 4 and 2 or 5 and 1. On day 3, embryos can be transferred to the uterus in a hyaluronan enriched medium (221).

Day 3 Embryos to the Blastocyst Stage

On day 3, before 8:30 am, label a 60 mm dish with the patient's name. Using a single-wrapped tip, rinse the tip, then place 6×25 μl drops of G2 into the

plate. Immediately cover with 9 mL of oil. Never prepare more than 2 plates at one time. Using a new tip for each drop, rinse the tip and then add a further 25 μl of medium to each original drop. Place the dish in the incubator and gently remove the lid and set on the side of the plate.

For each patient, set up one wash dish per 10 embryos. Place 1 mL of medium G2 into the center of an organ well dish. Place 2 mL of medium into the outer well. Place into the incubator. Dishes must gas in the incubator for a minimum of 4 hours. If working outside an isolette, use MOPS or HEPES buffered medium with amino acids. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage. Set up one sorting dish before 8:30 AM. Place 1 mL of medium G2 into the center of an organ well dish. Place 2 mL of medium into the outer well. Place immediately into the incubator. If working outside an isolette, use HEPES/MOPS buffered medium with amino acids. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage.

Moving embryos from G1 to G2 should occur between 10:00 AM and 2:00 PM Wash embryos in the organ well. Washing entails picking up the embryo 2–3 times and moving it around within the well. Transfer the embryos to the sorting dish and group like stage and quality embryos together. Rinse through the wash drops of medium and again place up to 5 embryos in each drop of G2. Return the dish to the incubator immediately. If working outside an isolette, use HEPES/MOPS buffered medium with amino acids in the sorting dish. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage.

The morning of day 5, embryos should be scored (Fig. 8) and the top one or two scoring embryos selected for transfer. Manipulation of blastocysts requires the use of a capillary bore of 275–300 μm. Transfers should be performed in a hyaluronan enriched medium (221). Any blastocysts not transferred can be cryopreserved. Should an embryo not have formed a blastocyst by day 5, it should be cultured in a fresh drop of G2 for 24 hours and assessed on day 6.

QUALITY CONTROL

Mouse Bioassay

The preimplantation mouse embryo is the most widely used bioassay for medium components, culture media, and equipment used in clinical IVF. Using mice for testing media for human embryos has been the focus of much discussion due to conflicting reports in the literature of its suitability as a bioassay (310,311). Fukuda et al. (312) reported that for the mouse, in vitro fertilization and the development of zygotes and 2-cell embryos in culture was positively correlated with the purity of the water source used in the preparation of media. In contrast, George et al. (313) and Silverman et al. (314)

found that media prepared with tap water could support adequate development of both 1-cell and 2-cell mouse embryos to the blastocyst stage respectively, compared to media prepared with ultrapure water. The apparent contradiction of these studies can be resolved by taking into account the different stages of development used at the start of culture, the types of media used, and the supplementation of medium with protein. Fukada et al. (312) used BWW, a "simple-type" medium, whereas Silverman et al. (314) used Ham's F-10. The latter medium contains amino acids, which may chelate any possible toxins present in the tap water, e.g., heavy metals. George et al. (313), included high levels of BSA in their zygote cultures to the blastocyst. Albumins can chelate potential embryotoxins and thereby mask the effect of any present in the culture medium (154,201). Furthermore, all studies used blastocyst development as the sole criterion for assessing embryo development. Blastocyst development is a poor indicator of embryo quality and does not reflect developmental potential (49,50,114). A far more sensitive and quantitative parameter is blastocyst cell number (38,274).

The sensitivity of mouse embryos to their environment is inversely proportional to the age of the embryo at recovery, i.e., 1-cell embryos are more susceptible to toxins in the medium than embryos collected at the 2-cell stage (315). Removal of the zona pellucida from the zygote may further increases the sensitivity of the embryo to the culture conditions (316), and endotoxins (317). Furthermore, the type of mouse bioassay performed depends upon the strain of mice used. Inbred strains and their F1 hybrids are less sensitive to their environment and, therefore, the embryos are collected at the zygote. Embryos from outbred strains of mice are more sensitive to environmental factors, however, such embryos exhibit the 2-cell block in culture media such as HTF and therefore are collected at the 2-cell stage. However, with the recent development of more optimized culture conditions, it is now possible to routinely culture such embryos from the zygote stage (16,19,24,25,40,50,105,130).

A practical mouse embryo bioassay is to culture the zygote for 96 hours in protein-free medium. The rationale for using protein-free medium is that serum or serum albumin can chelate toxins, such as heavy metal ions, present in the medium. The presence of proteins would therefore hide any potential detrimental effects of the medium. Multiple ovulations are induced by injecting four to six week old virgin females with 5–10 i.u. pregnant mares serum (PMS), followed 48 hours later with 5–10 i.u. human chorionic gonadotrophin (HCG). Females are placed with males immediately following the second injection and mating assessed the following morning by the presence of a vaginal plug. Embryos are cultured in groups of 10 in 20 μl drops of medium under an oil overlay at 37°C. Culture dishes should be set-up and allowed to equilibrate overnight in a 6% CO_2 atmosphere. To overcome any donor variation, embryos from each female should be

allocated equally to each treatment group. Around 10 AM on the day of plug, females are sacrificed and the oviducts excised and placed into warm collecting medium in a petri dish. The ampullary region of the oviduct is torn open close to the cumulus mass, which is then expelled under positive pressure into the medium. The cumulus is disaggregated by the addition of hyaluronidase (1 mg/mL) in collecting medium. After around 1 min, the cumulus disperses leaving denuded zygotes. The embryos are then washed twice in collecting medium and once in the culture medium and placed into culture. Zygotes are cultured in a protein free medium and cultured for 96 hours. Rather than a single end point of blastocyst development, it is important to determine appropriate on time development at set time points, and that there is no signs of necrosis in the blastocyst (this can be visualized easily on an inverted but not a stereo microscope, (258). Furthermore, it is most important that the cell number of the resulting blastocysts is determined. One can readily obtain 80% blastocyst development on day 5, but it is important also to note how embryos formed blastocysts in the afternoon of day 4 (i.e., on time development), and what the cell number is of the resultant blastocysts. There is a significant difference in the viability of blastocysts with 40 versus 80 cells.

Using such an approach to testing, one can pick up subtle problems that can exist with any media, or more typically with any contact supplies. It is important to note that just because a lot number of culture dishes has been approved safe for use in somatic cell tissue culture, does not automatically mean that it can support gametes or embryos.

With regards to alternative assays, such as hybridoma cell lines, although such cells in culture can be particularly sensitive to toxins in the medium, they are not embryonic cells and may therefore not detect potential embryo toxins. Although the mouse embryo bioassay does have a role in clinical IVF, at best it is only a test of the ability of the mouse embryo to develop. There is no guarantee that factors that do not affect the mouse will not be detrimental to the human. The ultimate quality control on all media for IVF is their ability to support human embryo growth. It is obviously unethical to use human embryos for this purpose. A possible solution would be the use of triploid embryos as an assay of media quality, as these cannot be replaced in the prospective mother. Therefore vigilant monitoring of embryo development within the IVF laboratory is not only essential, but should be considered as part of the laboratory's overall quality control system.

Sperm Bioassay

An alternative method to the mouse embryo bioassay is to use sperm motility as a method to detect potential embryo toxins. Hamster sperm bioassay has been used to assay media and medium components for potential

toxins (318–321). This assay utilizes both the number of motile sperm and degree of motility to determine the suitability of media to support embryo development. An advantage of this test is that it can be performed in four to six hours as opposed to the several days for the embryo bioassay. Unfortunately, however, there is little clinical data on the applicability of such tests.

REFERENCES

1. Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. J Embryol Exp Morphol 1970; 24:203.
2. Streffer C, van Beuningen D, Molls M, Zamboglou N, Schulz S. Kinetics of cell proliferation in the pre-implanted mouse embryo in vivo and in vitro. Cell Tissue Kinet 1980; 13:135.
3. Harlow GM, Quinn P. Development of preimplantation mouse embryos in vivo and in vitro. Aust J Biol Sci 1982; 35:187.
4. Edwards RG. Causes of early embryonic loss in human pregnancy. Hum Reprod 1986; 1:185.
5. Bernardini L, Alam V, Asch RH, Balmaceda JP. Pregnancy and implantation rates in normal replacement versus stimulated cycles. Hum Reprod 1993; 8:1938.
6. Hartshorne GM, Edwards RG. Early embryo development. In: Adashi EY, Rosenwaks Z, eds. Reproductive Endocrinology Surgery and Technology. Philadelphia: Lippincott-Raven Publishers, 1996:435.
7. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology 1998; 49:83.
8. Bavister BD. Culture of preimplantation embryos: facts and artifacts. Hum Reprod Update 1995; 1:91.
9. Gardner DK, Pool TB, Lane M. Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability. Semin Reprod Med 2000; 18:205.
10. Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update 2003; 9:557.
11. Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. Mol Reprod Dev 2003; 64:70.
12. Staessen C, Van den Abbeel E, Janssenswillen C, Devroey P, Van Steirteghem AC. Controlled comparison of Earle's balanced salt solution with Menezo B2 medium for human in vitro fertilization performance. Hum Reprod 1994; 9:1915.
13. Whitten WK, Biggers JD. Complete development in vitro of the pre-implantation stages of the mouse in a simple chemically defined medium. J Reprod Fertil 1968; 17:399.
14. Whittingham DG. Culture of mouse ova. J Reprod Fertil Suppl 1971; 14:7.
15. Edwards RG. Test-tube babies 1981. Nature 1981; 293:253.

16. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 1989; 86:679.
17. Lawitts JA, Biggers JD. Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. Mol Reprod Dev 1992; 31:189.
18. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985; 44:493.
19. Quinn P. Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. J Assist Reprod Genet 1995; 12:97.
20. Carrillo AJ, Lane B, Pridman DD, et al. Improved clinical outcomes for in vitro fertilization with delay of embryo transfer from 48 to 72 hours after oocyte retrieval: use of glucose- and phosphate-free media. Fertil Steril 1998; 69:329.
21. Whitten WK. The effect of progesterone on the development of mouse eggs in vitro. J Endocrinol 1957; 16:80.
22. Brinster RL. Studies on the development of mouse embryos in vitro. IV. Interaction of energy sources. J Reprod Fertil 1965; 10:227.
23. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. J Reprod Fertil 1990; 88:361.
24. Lawitts JA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. J Reprod Fertil 1991; 91:543.
25. Lawitts JA, Biggers JD. Culture of preimplantation embryos. Methods Enzymol 1993; 225:153.
26. Ham RG. An improved nutrient solution for diploid Chinese hamster and human cell lines. Exp Cell Res 1963; 29:515.
27. Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. Mol Reprod Dev 1995; 41:232.
28. Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. Biol Reprod 1993; 48:377.
29. Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM (AA) medium: is a two-step protocol necessary? Reprod Biomed Online 2002; 5: 133.
30. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. Fertil Steril 1996; 65:349.
31. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. Hum Reprod 1998; 13:3441.
32. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. Hum Reprod 1998; 13:991.
33. Lane M, Gardner DK. Regulation of ionic homeostasis by mammalian embryos. Semin Reprod Med 2000; 18:195.

34. Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. Biol Reprod 2000; 62:16.
35. Lane M. Mechanisms for managing cellular and homeostatic stress in vitro. Theriogenology 2001; 55:225.
36. Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod Fertil Dev 2005; 17:371.
37. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. Cell Biol Int 1994; 18:1163.
38. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Hum Reprod Update 1997; 3:367.
39. Pool TB. Recent advances in the production of viable human embryos in vitro. Reprod Biomed Online 2002; 4:294.
40. Gardner DK, Lane M. Development of viable mammalian embryos in vitro: evolution of sequential media. In: Cibelli R, Lanza K, Campbell AK, West MD, eds. Principles of Cloning. San Diego, Academic Press, 2002:187.
41. Barnes FL, Crombie A, Gardner DK, et al. Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum Reprod 1995; 10:3243.
42. Bertheussen K, Forsdahl F, Maltau JM. In vitro fertilization. New media for embryo culture to the blastocyst stage. In: Gomel V, Leung PCK, Eds. In Vitro Fertilization and Assisted Reproduction. Monduzzi Editoire, Bologna, 1997:199.
43. Behr B, Milki AA, Giudice LC, Moore D, Gebhardt J, Branch M. High yield blastocyst culture and transfer: A new approach using P1 and blastocyst medium in reduced O_2 environment. In: Proceedings of the 54th Annual Meeting of ASRM. San Francisco, 1998:S98.
44. Borland RM, Biggers JD, Lechene CP, Taymor ML. Elemental composition of fluid in the human Fallopian tube. J Reprod Fertil 1980; 58:479.
45. Borland RM, Hazra S, Biggers JD, Lechene CP. The elemental composition of the environments of the gametes and preimplantation embryo during the initiation of pregnancy. Biol Reprod 1977; 16:147.
46. Menezo Y, Testart J, Perrone D. Serum is not necessary in human in vitro fertilization, early embryo culture, and transfer. Fertil Steril 1984; 42:750.
47. Van Winkle LJ, Haghighat N, Campione AL. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. J Exp Zool 1990; 253:215.
48. Biggers JD, Lawitts JA, Lechene CP. The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro. Mol Reprod Dev 1993; 34:380.
49. Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: role of medium composition. Hum Reprod 1993; 8:288.
50. Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. J Reprod Fertil 1997; 109:153.
51. Wales RG. Effects of ions on the development of the pre-implantation mouse embryo in vitro. Aust J Biol Sci 1970; 23:421.
52. Bavister BD, Golden M. Alteration of extracellular cation concentrations and ratios in culture medium does not affect first cleavage division of hamster

zygotes in vitro nor overcome the 'two-cell block'. Reprod Fertil Dev 1989; 1:231.
53. McKiernan SH, Bavister BD. Environmental variables influencing in vitro development of hamster 2-cell embryos to the blastocyst stage. Biol Reprod 1990; 43:404.
54. Roblero LS, Guadarrama A, Ortiz ME, Fernandez E, Zegers-Hochschild F. High potassium concentration and the cumulus corona oocyte complex stimulate the fertilizing capacity of human spermatozoa. Fertil Steril 1990; 54:328.
55. Roblero LS, Riffo MD. High potassium concentration improves preimplantation development of mouse embryos in vitro. Fertil Steril 1986; 45:412.
56. Erbach GT, Lawitts JA, Papaioannou VE, Biggers JD. Differential growth of the mouse preimplantation embryo in chemically defined media. Biol Reprod 1994; 50:1027.
57. Wiley LM, Yamami S, Van Muyden D. Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development in vitro. Fertil Steril 1986; 45:111.
58. Dumoulin JC, Evers JL, Michiels AH, et al. Modulation of embryonic Na(+)-K(+)-ATPase activity and mouse preimplantation development in vitro in media containing high concentrations of potassium. Mol Reprod Dev 1993; 36:320.
59. Anbari K, Schultz RM. Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos in vitro. Mol Reprod Dev 1993; 35:24.
60. Ducibella T, Anderson E. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. Dev Biol 1975; 47:45.
61. Lane M, Boatman DE, Albrecht RM, Bavister BD. Intracellular divalent cation homeostasis and developmental competence in the hamster preimplantation embryo. Mol Reprod Dev 1998; 50:443.
62. Lane M, Bavister BD. Calcium homeostasis in early hamster preimplantation embryos. Biol Reprod 1998; 59:1000.
63. Rout UK, Krawetz SA, Armant DR. Ethanol-induced intracellular calcium mobilization rapidly alters gene expression in the mouse blastocyst. Cell Calcium 1997; 22:463.
64. Reed L, Gardner DK. Phosphate-free culture media impairs ICM development and alters blastocyst metabolism. Fertil Steril 2005; 84:S379.
65. Brinster RL. Studies on the development of mouse embryos in vitro. I. The effect of osmolarity and hydrogen ion concentration. J Exp Zool 1965; 158:49.
66. Bavister BD, McKiernan SH. Regulation of hamster embryo development in vitro by amino acids. In: Bavister BD, ed. Preimplantation Embryo Development. New York: Springer-Verlag, 1992:57.
67. Dawson KM, Collins JL, Baltz JM. Osmolarity-dependent glycine accumulation indicates a role for glycine as an organic osmolyte in early preimplantation mouse embryos. Biol Reprod 1998; 59:225.
68. Nichol R, Hunter RH, Gardner DK, Leese HJ, Cooke GM. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. J Reprod Fertil 1992; 96:699.

69. Leese HJ, Barton AM. Production of pyruvate by isolated mouse cumulus cells. J Exp Zool 1985; 234:231.
70. Leese HJ, Hooper MA, Edwards RG, Ashwood-Smith MJ. Uptake of pyruvate by early human embryos determined by a non-invasive technique. Hum Reprod 1986; 1:181.
71. Hardy K, Hooper MA, Handyside AH, Rutherford AJ, Winston RM, Leese HJ. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. Hum Reprod 1989; 4:188.
72. Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. Hum Reprod 1990; 5:104.
73. Butcher L, Coates A, Martin KL, Rutherford AJ, Leese HJ. Metabolism of pyruvate by the early human embryo. Biol Reprod 1998; 58:1054.
74. Gardner DK, Lane M, stevens J, Schoolcraft WB. Non-invasive assessment of human nutrient consumption as a measure of developmental potential. Fertil Steril 2001; 76:1175.
75. Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. J Reprod Fertil 1984; 72:9.
76. Gardner DK, Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. Hum Reprod 1986; 1:25.
77. Gardner DK, Lane M, Batt P. Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed in vivo. Mol Reprod Dev 1993; 36:313.
78. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci USA 1967; 58:560.
79. Conaghan J, Handyside AH, Winston RM, Leese HJ. Effects of pyruvate and glucose on the development of human preimplantation embryos in vitro. J Reprod Fertil 1993; 99:87.
80. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. J Biol Chem 2005; 280:18361.
81. Cross PC, Brinster RL. The sensitivity of one-cell mouse embryos to pyruvate and lactate. Exp Cell Res 1973; 77:57.
82. Pomp D, Crister ES, Rutledge JJ. Lower sodium lactate in Whitten's medium improves in vitro developmental capacity of one-cell mouse embryos. Theriogenology 1988; 29:1019.
83. Wales RG, Whittingham. The metabolism of specifically labelled lactate and pyruvate by two-cell mouse embryos. J Reprod Fertil 1973; 33:207.
84. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. Mol Reprod Dev 1998; 50:434.
85. Brinster RL, Thomson JL. Development of eight-cell mouse embryos in vitro. Exp Cell Res 1966; 42:308.
86. Barbehenn EK, Wales RG, Lowry OH. The explanation for the blockade of glycolysis in early mouse embryos. Proc Natl Acad Sci USA 1974; 71:1056.
87. Barbehenn EK, Wales RG, Lowry OH. Measurement of metabolites in single preimplantation embryos: a new means to study metabolic control in early embryos. J Embryol Exp Morphol 1978; 43:29.

88. Biggers JD, Gardner DK, Leese HJ. Control of carbohydrate metabolism in preimplantation mammalian embryos. In: Rosenblum IY, Heyner S, eds. Growth Factors in Mammalian Embryos. Boca Raton: CRC Press, 1989:19.
89. Schini SA, Bavister BD. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol Reprod 1988; 39:1183.
90. Thompson JG, Simpson AC, Pugh PA, Tervit HR. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol Reprod Dev 1992; 31:253.
91. Takahashi Y, First NL. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992; 37:963.
92. Rosenkrans CF Jr., Zeng GQ, MCNamara GT, Schoff PK, First NL. Development of bovine embryos in vitro as affected by energy substrates. Biol Reprod 1993; 49:459.
93. Matsuyama K, Miyakoshi K, Fukui Y. Effect of glucose levels during in vitro culture in synthetic oviduct fluid medium on in vitro development of bovine oocytes matured and fertilized in vitro. Theriogenology 1993; 40:595.
94. Koobs DH. Phosphate mediation of the Crabtree and Pasteur effects. Science 1972; 178:127.
95. Seshagiri PB, Bavister BD. Glucose and phosphate inhibit respiration and oxidative metabolism in cultured hamster eight-cell embryos: evidence for the "Crabtree effect". Mol Reprod Dev 1991; 30:105.
96. Gardner DK, Lane M. The 2-cell block in CF1 mouse embryos is associated with an increase in glycolysis and a decrease in tricarboxylic acid (TCA) cycle activity: alleviation of the 2-cell block is associated with the restoration of in vivo metabolic pathway activities. Biol Reprod 1993; 49(suppl 1):152.
97. Gardner DK. Embryo Development and culture techniques. In: Clark J, ed. Animal Breeding: Technology for the 21st Century. London: Harwood Academic, 1998:13.
98. Leese HJ. Metabolism of the preimplantation mammalian embryo. Oxf Rev Reprod Biol 1991; 13:35.
99. Edwards LE, Gardner DK. Characterisation of Hexokinasekinetics in the preimplantation mouse embryo. Proc Fert Soc Aus, Melbourne, Vic, 1995:28.
100. Wilson JE. Hexokinases. Rev Physiol Biochem Pharmacol 1995; 126:65.
101. Barnett DK, Bavister BD. Inhibitory effect of glucose and phosphate on the second cleavage division of hamster embryos: is it linked to metabolism? Hum Reprod 1996; 11:177.
102. Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biol Reprod 1994; 50:390.
103. Lane M, Gardner DK. Inhibiting 3-phosphoglycerate kinase by EDTA stimulates the development of the cleavage stage mouse embryo. Mol Reprod Dev 2001; 60:233.
104. Aoki K, Nakamura M, Namiki H, Okinaga S, Arai K. The effect of glucose and phosphate on mouse two-cell embryos to develop in vitro. Zool Sci 1990; 7:973.

105. Gardner DK, Lane M. Alleviation of the ‘2-cell block’ and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. Hum Reprod 1996; 11:2703.
106. Ludwig TE, Lane M, Bavister BD. Increased fetal development after transfer of hamster embryos cultured with glucose. Biol Reprod 1998; 58(suppl 1):306.
107. Aghayan M, Rao LV, Smith RM, et al. Developmental expression and cellular localization of glucose transporter molecules during mouse preimplantation development. Development 1992; 115:305.
108. Dan-Goor M, Sasson S, Davarashvili A, Almagor M. Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. Hum Reprod 1997; 12:2508.
109. Gardner DK, Leese HJ. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. Development 1988; 104:423.
110. Hogan A, Heyner S, Charron MJ, et al. Glucose transporter gene expression in early mouse embryos. Development 1991; 113:363.
111. Rogers PAW, Murphy CR, Gannon BJ. Changes in the spatial organisation of the uterine vasculature during implantation in the rat. J Reprod Fertil 1982; 65:211.
112. Rogers PAW, Murphy CR, Gannon BJ. Absence of capillaries in the endometrium surrounding the implanting rat blastocyst. Micron 1982; 13:373.
113. Gardner DK. The Nutrition and Energy Metabolism of the Preimplantation Mouse Embryo, Ph.D., University of York, York, UK, 1987.
114. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod 1996; 11:1975.
115. Perkins JL, Goode L. Free amino acids in the oviduct fluid of the ewe. J Reprod Fertil 1967; 14:309.
116. Menezo Y. Amino constituents of tubal and uterine fluids of the eostrous ewe: comparison with blood serum and ram seminal fluid. In: Hafez ES, Thibault C, eds. The Biology of Spermatozoa. New York: Basel Press, 1972:174.
117. Casslen BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. J Reprod Med 1987; 32:181.
118. Miller JG, Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. Biol Reprod 1987; 36:125.
119. Van Winkle LJ. Amino acid transport in developing animal oocytes and early conceptuses. Biochim Biophys Acta 1988; 947:173.
120. Schultz GA, Kaye PL, McKay DJ, Johnson MH. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. J Reprod Fertil 1981; 61:387.
121. Chatot CL, Tasca RJ, Ziomek CA. Glutamine uptake and utilization by preimplantation mouse embryos in CZB medium. J Reprod Fertil 1990; 89:335.
122. Gardner DK, Clarke RN, Lechene CP, Biggers JD. Development of a noninvasive ultramicrofluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos. Gamete Res 1989; 24:427.
123. Eagle H. Amino acid metabolism in mammalian cell cultures. Science 1959; 130:432.
124. Dumoulin JC, Evers JL, Bakker JA, Bras M, Pieters MH, Geraedts JP. Temporal effects of taurine on mouse preimplantation development in vitro. Hum Reprod 1992; 7:403.

125. Dumoulin JC, Evers JL, Bras M, Pieters MH, Geraedts JP. Positive effect of taurine on preimplantation development of mouse embryos in vitro. J Reprod Fertil 1992; 94:373.
126. Bavister BD. Studies on the developmental blocks in cultured hamster embryos. In: Bavister BD, ed. The Mammalian Preimplantation Embryo. New York: Plenum, 1987:219.
127. McKiernan SH, Clayton MK, Bavister BD. Analysis of stimulatory and inhibitory amino acids for development of hamster one-cell embryos in vitro. Mol Reprod Dev 1995; 42:188.
128. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod 1995; 53:1385.
129. Lane M, Gardner DK. Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes in vitro. J Assist Reprod Genet 1997; 14:398.
130. Lane M, Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. J Reprod Fertil 1994; 102:305.
131. Gardner DK, Lane M. Towards a single embryo transfer. Reprod Biomed Online 2003; 6:470.
132. Steeves TE, Gardner DK. Temporal and differential effects of amino acids on bovine embryo development in culture. Biol Reprod 1999; 61:731.
133. Bavister BD, Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. Mol Reprod Dev 1990; 25:45.
134. Gardner DK, Lane M. Culture Systems for human embryo. In: Gardner DK, Weissman A, Howles C, Shoham Z, eds. Textbook of Assisted Reproductive Techniques Laboratory and Clinical Perspectives, 2nd ed. Taylor and Francis, London and New York, 2004:211.
135. Li J, Foote RH. Effect of inositol and glycine with increasing sodium chloride and constant osmolality on development of rabbit embryos. J Assist Reprod Genet 1995; 12:141.
136. Mehta TS, Kiessling AA. Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. Biol Reprod 1990; 43:600.
137. Zhang X, Armstrong DT. Presence of amino acids and insulin in a chemically defined medium improves development of 8-cell rat embryos in vitro and subsequent implantation in vivo. Biol Reprod 1990; 42:662.
138. Devreker F, Winston RM, Hardy K. Glutamine improves human preimplantation development in vitro. Fertil Steril 1998; 69:293.
139. Lane M, Gardner DK. Removal of embryo-toxic ammonium from the culture medium by in situ enzymatic conversion to glutamate. J Exp Zool 1995; 271:356.
140. Gardner DK, Stilley KS, Lane M. High protein diet inhibits inner cell mass formation and increases apoptosis in mouse blastocysts developed in vivo by increasing the levels of ammonium in the reproductive tract. Reprod, Fertil Dev 2004; 16:190.
141. Lane M, Hewitt EA, Gardner DK. Ammonium alters gene expression and imprinting of H19 in cultured mouse embryos. In: Proc Serono Symp, 2003.

142. Zander DL, Thompson JG, Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. Biol Reprod 2006; 74:288.
143. Sinawat S, Hsaio WC, Flockhart JH, Kaufman MH, Keith J, West JD. Fetal abnormalities produced after preimplantation exposure of mouse embryos to ammonium chloride. Hum Reprod 2003; 18:2157.
144. Biggers JD, McGinnis LK, Lawitts JA. Enhanced effect of glycyl-L-glutamine on mouse preimplantation embryos in vitro. Reprod Biomed Online 2004; 9:59.
145. Virant-Klun I, Tomazevic T, Vrtacnik-Bokal E, Vogler A, Krsnik M, Meden-Vrtovec H. Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. Fertil Steril 2006; 85:526.
146. Bolton VN, Hawes SM, Taylor CT, Parsons JH. Development of spare human preimplantation embryos in vitro: an analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst. J In Vitro Fert Embryo Transf 1989; 6:30.
147. Hardy K. Bavister BD, ed. Preimplantation Embryo Development. New York: Springer-Verlag, 1993:184.
148. Kane MT. The effects of water-soluble vitamins on the expansion of rabbit blastocysts in vitro. J Exp Zool 1988; 245:220.
149. Tsai FC, Gardner DK. Nicotinamide, a component of complex culture media, inhibits mouse embryo development in vitro and reduces subsequent developmental potential after transfer. Fertil Steril 1994; 61:376.
150. Loutradis D, John D, Kiessling AA. Hypoxanthine causes a 2-cell block in random-bred mouse embryos. Biol Reprod 1987; 37:311.
151. Downs SM, Dow MP. Hypoxanthine-maintained two-cell block in mouse embryos: dependence on glucose and effect of hypoxanthine phosphoribosyltransferase inhibitors. Biol Reprod 1991; 44:1025.
152. Nureddin A, Epsaro E, Kiessling AA. Purines inhibit the development of mouse embryos in vitro. J Reprod Fertil 1990; 90:455.
153. Abramczuk J, Solter D, Koprowski H. The beneficial effect EDTA on development of mouse one-cell embryos in chemically defined medium. Dev Biol 1977; 61:378.
154. Fissore RA, Jackson KV, Kiessling AA. Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. Biol Reprod 1989; 41:835.
155. Mehta TS, Kiessling AA. The developmental potential of mouse embryos conceived in Ham's F-10 medium containing ethylenediaminetetraacetic acid. Fertil Steril 1993; 60:1088.
156. Bertheussen K. Growth of cells in a new defined protein-free medium. Cytotechnology 1993; 11:219.
157. Hoshi M, Toyoda Y. Effect of EDTA on the preimplantation development of mouse embryos fertilized in vitro. Jpn J Zootechnol Sci 1985; 56:931.
158. Gardner DK, Lane MW, Lane M. Bovine blastocyst cell number is increased by culture with EDTA for the first 72 hours of development from zygote. Theriogenology 1997; 47:278.
159. Hewitson LC, Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. J Exp Zool 1993; 267:337.

160. Nasr-Esfahani M, Johnson MH, Aitken RJ. The effect of iron and iron chelators on the in-vitro block to development of the mouse preimplantation embryo: BAT6 a new medium for improved culture of mouse embryos in vitro. Hum Reprod 1990; 5:997.
161. Nasr-Esfahani MH, Johnson MH. How does transferrin overcome the in vitro block to development of the mouse preimplantation embryo? J Reprod Fertil 1992; 96:41.
162. Noda Y. Embryo development in vitro. Assist Reprod Rev 1992; 2:9.
163. Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T. Involvement of superoxide radicals in the mouse two-cell block. Mol Reprod Dev 1991; 28:356.
164. Umaoka Y, Noda Y, Narimoto K, Mori T. Effects of oxygen toxicity on early development of mouse embryos. Mol Reprod Dev 1992; 31:28.
165. Payne SR, Munday R, Thompson JG. Addition of superoxide dismutase and catalase does not necessarily overcome developmental retardation of one-cell mouse embryos during in-vitro culture. Reprod Fertil Dev 1992; 4:167.
166. Lindenau A, Fischer B. Effect of oxygen concentration in the incubator's gas phase on the development of cultured preimplantation rabbit embryos. Theriogenology 1994; 41:889.
167. Liu Z, Foote RH. Effects of amino acids on the development of in-vitro matured/in vitro fertilization bovine embryos in a simple protein-free medium. Hum Reprod 1995; 10:2985.
168. Legge M, Sellens MH. Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. Hum Reprod 1991; 6:867.
169. Nasr-Esfahani MH, Johnson MH. Quantitative analysis of cellular glutathione in early preimplantation mouse embryos developing in vivo and in vitro. Hum Reprod 1992; 7:1281.
170. Gardiner CS, Salmen JJ, Brandt CJ, Stover SK. Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. Biol Reprod 1998; 59:431.
171. Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A. Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. Biol Reprod 1993; 49:228.
172. Lim J, Liou SS, Hansel W. Intracytoplasmic glutathione concentration and beta-mercaptoethanol in preimplantation development of bovine embryo. Theriogenology 1996; 46:429.
173. de Matos DG, Furnus CC, Moses DF, Martinez AG, Matkovic M. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. Mol Reprod Dev 1996; 45:451.
174. de Matos DG, Nogueira D, Cortvrindt R, et al. Capacity of adult and prepubertal mouse oocytes to undergo embryo development in the presence of cysteamine. Mol Reprod Dev 2003; 64:214.
175. Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993; 49:89.
176. Caamano JN, Ryoo ZY, Youngs CR. Promotion of development of bovine embryos produced in vitro by addition of cysteine and beta-mercaptoethanol to a chemically defined culture system. J Dairy Sci 1998; 81:369.

177. Andrae U, Singh J, Ziegler-Skylakakis K. Pyruvate and related alpha-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. Toxicol Lett 1985; 28:93.
178. Kouridakis K, Gardner DK. Pyruvate in embryo culture embryo media acts as an antioxidant. In: Proc Fert Soc Aus. Melbourne, Victoria, 1995:29.
179. O'Fallon JV, Wright RW. Pyruvate revisited: a non-metabolic role for pyruvate in preimplantation embryo development. Theriogenology 1995; 43:288.
180. Li J, Foote RH, Simkin M. Development of rabbit zygotes cultured in protein-free medium with catalase, taurine, or superoxide dismutase. Biol Reprod 1993; 49:33.
181. Lane M, Maybach JM, Gardner DK. Addition of ascorbate during cryopreservation stimulates subsequent embryo development. Hum Reprod 2002; 17: 2686.
182. Magli MC, Gianaroli L, Fiorentino A, Ferraretti AP, Fortini D, Panzella S. Improved cleavage rate of human embryos cultured in antibiotic-free medium. Hum Reprod 1996; 11:1520.
183. Otoi T, Tachikawa S, Kondo S, Suzuki T. Effect of antibiotics treatment of in vitro fertilized bovine embryos to remove adhering bacteria. J Vet Med Sci 1992; 54:763.
184. Barnes D, Sato G. Methods for growth of cultured cells in serum-free medium. Anal Biochem 1980; 102:255.
185. Barnes D, Sato G. Serum-free cell culture: a unifying approach. Cell 1980; 22:649.
186. Damewood MD, Hesla JS, Schlaff WD, Hubbard M, Gearhart JD, Rock JA. Effect of serum from patients with minimal to mild endometriosis on mouse embryo development in vitro. Fertil Steril 1990; 54:917.
187. Abu-Musa A, Takahashi K, Kitao M. The effect of serum obtained before and after treatment for endometriosis on in vitro development of two-cell mouse embryos. Fertil Steril 1992; 57:1098.
188. Dokras A, Sargent IL, Redman CW, Barlow DH. Sera from women with unexplained infertility inhibit both mouse and human embryo growth in vitro. Fertil Steril 1993; 60:285.
189. Miller KA, Pittaway DE, Deaton JL. The effect of serum from infertile women with endometriosis on fertilization and early embryonic development in a murine in vitro fertilization model. Fertil Steril 1995; 64:623.
190. Deaton JL, Dempsey RA, Miller KA. Serum from women with polycystic ovary syndrome inhibits fertilization and embryonic development in the murine in vitro fertilization model. Fertil Steril 1996; 65:1224.
191. Leese HJ. The formation and function of oviduct fluid. J Reprod Fertil 1988; 82:843.
192. Dorland M, Gardner DK, Trounson A. Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos[abstr ser]. Reprod Fertil 1994; 13:70.
193. Khosla S, Dean W, Brown D, reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 2001; 64:918.
194. Walker SK, Heard TM, Seamark RF. In vitro culture of sheep embryos without co-culture: success and perspectives. Theriogenology 1992; 37:111.

195. Gardner DK. Development of serum-free media for the culture and transfer of human blastocysts. Hum Reprod 1998; 13(suppl 4):218.
196. Young LE, Fernandes K, McEvoy TG, et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001; 27:153.
197. Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. Reprod Fertil Dev 2005; 17:361.
198. Killian JK, Nolan CM, Wylie AA, et al. Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. Hum Mol Genet 2001; 10:1721.
199. Menezo YJ, Chouteau J, Torello J, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. Fertil Steril 1999; 72:221.
200. Holst N, Bertheussen K, Forsdahl F, Hakonsen MB, Hansen LJ, Nielsen HI. Optimization and simplification of culture conditions in human in vitro fertilization (IVF) and preembryo replacement by serum-free media. J In Vitro Fert Embryo Transf 1990; 7:47.
201. Flood LP, Shirley B. Reduction of embryotoxicity by protein in embryo culture media. Mol Reprod Dev 1991; 30:226.
202. Ashwood-Smith MJ, Hollands P, Edwards RG. The use of Albuminar 5 as a medium supplement in clinical IVF. Hum Reprod 1989; 4:702.
203. Khan I, Staessen C, Devroey P, Van Steirteghem. Human serum albumin versus serum: a comparative study on embryo transfer medium. Fertil Steril 1991; 56:98.
204. Staessen C, Van den Abbeel E, Carle M, Khan I, Devroey P, Van Steirteghem AC. Comparison between human serum and Albuminar-20 (TM) supplement for in-vitro fertilization. Hum Reprod 1990; 5:336.
205. Benadiva CA, Kuczynski-Brown B, Maguire TG, Mastoianni L Jr, Flickinger GL. Bovine serum albumin (BSA) can replace patient serum as a protein source in an in vitro fertilization (IVF) program. J In Vitro Fert Embryo Transf 1989; 6:164.
206. Huisman GJ, Lo-A-Njoe NM, Alberda AT, Leerentveld RA, Verhoeff A, Zeilmaker GH. Comparison of results obtained with human serum and a protein solution as a supplement for in vitro fertilization culture medium. Fertil Steril 1992; 58:637.
207. Adler A, Reing AM, Bedford JM, Alikani M, Cohen J. Plasmanate as a medium supplement for in vitro fertilization. J Assist Reprod Genet 1993; 10:67.
208. Pool TB, Martin JE. High continuing pregnancy rates after in vitro fertilization-embryo transfer using medium supplemented with a plasma protein fraction containing alpha- and beta-globulins. Fertil Steril 1994; 61:714.
209. Weathersbee PS, Pool TB, Ord T. Synthetic serum substitute (SSS): a globulin-enriched protein supplement for human embryo culture. J Assist Reprod Genet 1995; 12:354.
210. Desai NN, Sheean LA, Martin D, et al. Clinical experience with synthetic serum substitute as a protein supplement in IVF culture media: a retrospective study. J Assist Reprod Genet 1996; 13:23.
211. Tucker KE, Hurst BS, Guadagnoli S, et al. Evaluation of synthetic serum substitute versus serum as protein supplementation for mouse and human embryo culture. J Assist Reprod Genet 1996; 13:32.

212. Hunter RH. Modulation of gamete and embryonic microenvironments by oviduct glycoproteins. Mol Reprod Dev 1994; 39:176.
213. Hanson RW, Ballard FJ. Citrate, pyruvate, and lactate contaminants of commercial serum albumin. J Lipid Res 1968; 9:667.
214. Gray CW, Morgan PM, Kane MT. Purification of an embryotrophic factor from commercial bovine serum albumin and its identification as citrate. J Reprod Fertil 1992; 94:471.
215. Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod Fertil Dev 1991; 3:601.
216. McKiernan SH, Bavister BD. Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. In Vitro Cell Dev Biol 1992; 28A:154.
217. Kane MT. Variability in different lots of commercial bovine serum albumin affects cell multiplication and hatching of rabbit blastocysts in culture. J Reprod Fertil 1983; 69:555.
218. Bungum M, Humaidan P, Bungum L. Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study. Reprod Biomed Online 2002; 4:233.
219. Bavister BD, Kinsey DL, Lane M, Gardner DK. Recombinant human albumin supports hamster in-vitro fertilization. Hum Reprod 2003; 18:113.
220. Bavister BD. Substitution of a synthetic polymer for protein in a mammalian gamete culture system. J Exp Zool 1981; 217:45.
221. Gardner DK, Lane M, Rodriguez-Martinez H. Fetal development after transfer in increased by replacing protein with the glycoaminoglycan hyaluronate for embryo culture. Hum Reprod 1999; 14:2575.
222. Stojkovic M, Kolle S, Peinl S, et al. Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen-thawed bovine embroyos produced in vitro. Reproduction 2002; 124:141.
223. Kano K, Miyano T, Kato S. Effects of glycosaminoglycans on the development of in vitro-matured and -fertilized porcine oocytes to the blastocyst stage in vitro. Biol Reprod 1998; 58:1226.
224. Behzad F, Behzad F, Seif MW, Campbell S, Aplin JD. Expression of two isoforms of CD44 in human endometrium. Biol Reprod 1994; 51:739.
225. Wu JT. Metabolism of progesterone by preimplantation mouse blastocysts in culture. Biol Reprod 1987; 36:549.
226. Edirisinghe WR, Wales RG. Effect of parenteral administration of oestrogen and progesterone on the glycogen metabolism of mouse morulae–early blastocysts in vivo. J Reprod Fertil 1984; 72:67.
227. Edirisinghe WR, Wales RG. Influence of environmental factors on the metabolism of glucose by preimplantation mouse embryos in vitro. Aust J Biol Sci 1985; 38:411.
228. Khurana NK, Wales RG. Effects of oestradiol and progesterone on the metabolism of [U-14C]glucose by mouse morulae and early blastocysts in vitro. J Reprod Fertil 1987; 79:267.
229. Khurana NK, Wales RG. Effects of prostaglandins E-2 and F-2 alpha on the metabolism of [U-14C]glucose by mouse morulae-early blastocysts in vitro. J Reprod Fertil 1987; 79:275.

230. Valbuena D, Martin J, de Pablo JL, Remohi J, Pellicher A, Simon C. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. Fertil Steril 2001; 76:962.
231. Karagenc L, Lane M, Gardner DK. Oestradiol, cyclodextrin-encapsulated 17beta-oestradiol and the oestradiol solubilizer 2-hydroxypropyl-beta-cyclodextrin all impair preimplantation mouse embryo development. Reprod Biomed Online 2004; 9:280.
232. Yohkaichiya T, Fukaya T, Hoshiai H, Yajima A. Improvement of mouse embryo development in vitro by prolactin. Tohoku J Exp Med 1988; 155:241.
233. Lavranos TC, Seamark RF. Addition of steroids to embryo-uterine monolayer co-culture enhances embryo survival and implantation in vitro. Reprod Fertil Dev 1989; 1:41.
234. Kaye PL, Bell KL, Beebe LF, Dunglison GF, Gardner HG, Harvey MB. Insulin and the insulin-like growth factors (IGFs) in preimplantation development. Reprod Fertil Dev 1992; 4:373.
235. Schultz GA, Heyner S. Growth factors in preimplantation mammalian embryos. Oxf Rev Reprod Biol 1993; 15:43.
236. Adamson ED. Activities of growth factors in preimplantation embryos. J Cell Biochem 1993; 53:280.
237. Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR, Shultz GA. Roles of growth factors during peri-implantation development. Hum Reprod 1995; 10:712.
238. Stewart CL. Leukaemia inhibitory factor and the regulation of pre-implantation development of the mammalian embryo. Mol Reprod Dev 1994; 39:233.
239. Kane MT, Morgan PM, Coonan C. Peptide growth factors and preimplantation development. Hum Reprod Update 1997; 3:137.
240. Martin KL, Barlow DH, Sargent IL. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. Hum Reprod 1998; 13:1645.
241. Lighten AD, Moore GE, Winston RM, Hardy K. Routine addition of human insulin-like growth factor-I ligand could benefit clinical in vitro fertilization culture. Hum Reprod 1998; 13:3144.
242. Smotrich DB, Stillman RJ, Widra EA, et al. Immunocytochemical localization of growth factors and their receptors in human pre-embryos and Fallopian tubes. Hum Reprod 1996; 11:184.
243. Chia CM, Winston RM, Handyside AH. EGF, TGF-alpha and EGFR expression in human preimplantation embryos. Development 1995; 121:299.
244. Dunglison GF, Barlow DH, Sargent IL. Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. Hum Reprod 1996; 11:191.
245. Krussel JS, Huang HY, Simon et al. Single blastomeres within human preimplantation embryos express different amounts of messenger ribonucleic acid for beta-actin and interleukin-1 receptor type I. J Clin Endocrinol Metab 1998; 83:953.
246. Ryan JP, O'Neill C. Embryo-derived and maternal factors associated with developmental potential and viability of preimplantation mammalian embryos. In: Van Blerkom J, ed. The Biological Basis of Early Human Reproductive Failure. New York: Oxford University Press, 1994:375.

247. Quinn P, Wales RG. Growth and metabolism of preimplantation mouse embryos cultured in phosphate-buffered medium. J Reprod Fertil 1973; 35:289.
248. Minhas BS, Randall GW, Dobson MG, Robertson JL. A limited exposure of murine oocytes and zygotes to phosphate buffered saline retards development in vitro. Theriogenology 1989; 31:229.
249. Mahadevan MM, Fleetham J, Church RB, Taylor PJ. Growth of mouse embryos in bicarbonate media buffered by carbon dioxide, hepes, or phosphate. J In Vitro Fert Embryo Transf 1986; 3:304.
250. Good NE, Winger GC, Winter W, Connolly TN, Izawa S, Sing RM. Hydrogen ion buffers for biological research. Biochemistry 1966; 5:467.
251. Quinn P, Barros C, Whittingham DG. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J Reprod Fertil. 1982; 66:161.
252. Behr BR, Stratton CJ, Foote WD, Knutzen V, Sher G. In vitro fertilization (IVF) of mouse ova in HEPES-buffered culture media. J In Vitro Fert Embryo Transf 1990; 7:9.
253. Hardy K, Robinson FM, Paraschos T, Wicks R, Franks S, Winston RM. Normal development and metabolic activity of preimplantation embryos in vitro from patients with polycystic ovaries. Hum Reprod 1995; 10:2125.
254. Simon C, Garcia Velasco JJ, Valbuena D, et al. Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a follicle-stimulating hormone step-down regimen. Fertil Steril 1998; 70:234.
255. Van der Auwera I, Pijnenborg R, Koninckx PR. The influence of in-vitro culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation. Hum Reprod 1999; 14:2570.
256. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. Hum Reprod 2001; 16:221.
257. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 2000; 127:4195.
258. Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization. Semin Reprod Med 2005; 23:319.
259. Mastroianni L Jr., Jones R. Oxygen tension within the rabbit fallopian tube. J Reprod Fertil 1965; 9:99.
260. Ross RN, Graves CN. O_2 levels in female rabbit reproductive tract. J Anim Sci 1974; 39:994.
261. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil 1993; 99:673.
262. Maas DH, Storey BT, Mastroianni L Jr. Oxygen tension in the oviduct of the rhesus monkey (*Macaca mulatta*). Fertil Steril 1976; 27:1312.
263. Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. J Reprod Fertil 1990; 89:573.
264. Pabon JE Jr., Findley WE, Gibbons WE. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil Steril 1989; 51:896.

265. Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos in vitro. J Exp Zool 1978; 206:73.
266. Li J, Foote RH. Culture of rabbit zygotes into blastocysts in protein-free medium with one to twenty percent oxygen. J Reprod Fertil 1993; 98:163.
267. Graves CN, Biggers JD. Carbon dioxide fixation by mouse embryos prior to implantation. Science 1970; 167:1506.
268. Garris DR. Uterine blood flow, pH, and pCO_2 during nidation in the guinea pig: ovarian regulation. Endocrinology 1984; 114:1219.
269. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. Biol Reprod 1987; 36:1155.
270. Halliden K, Li J, Carney EW, Foote RH. Increasing carbon dioxide from five percent to ten percent improves rabbit blastocyst development from cultured zygotes. Mol Reprod Dev 1992; 33:276.
271. Farrell PB, Foote RH. Beneficial effects of culturing rabbit zygotes to blastocysts in 5% oxygen and 10% carbon dioxide. J Reprod Fertil 1995; 103;127.
272. Maas DH, Storey BT, Mastroianni L Jr. Hydrogen ion and carbon dioxide content of the oviductal fluid of the rhesus monkey (*Macaca mulatta*). Fertil Steril 1977; 28:981.
273. Dale B, Menezo Y, Cohen J, DiMatteo L, Wilding M. Intracellular pH regulation in the human oocyte. Hum Reprod 1998; 13:964.
274. FitzGerald-Scott L, Sundaram SG, Smith S. The relevance and use of mouse embryos bioassays for quality control in an assisted reproductive technology program. Fertil Steril 1993; 60:559.
275. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. Dev Biol 1999; 208:244.
276. Zhao Y, Baltz JM. Bicarbonate/chloride exchange and intracellular pH throughout preimplantation mouse embryo development. Am J Physiol 1996; 271:C1512.
277. Phillips KP, Baltz JM. Intracellular pH regulation by HCO_3^-/Cl^- exchanger activity appears following fertilization in the mouse. Dev Biol 1999; 208:392.
278. Fischer B, Schumacher A, Hegele-Hartung C, Beier HM. Potential risk of light and room temperature exposure to preimplantation embryos. Fertil Steril 1988; 50:938.
279. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. Fertil Steril 1990; 54:102.
280. Umaoka Y, Noda Y, Nakayama T, Narimoto K, Mori T, Iritani A. Effect of visual light on in vitro embryonic development in the hamster. Theriogenology 1992; 38:1043.
281. Schumacher A, Fischer B. Influence of visible light and room temperature on cell proliferation in preimplantation rabbit embryos. J Reprod Fertil 1988; 84:197.
282. Hirao Y, Yanagimachi R. Detrimental effect of visible light on meiosis of mammalian eggs in vitro. J Exp Zool 1978; 206:365.
283. Noda Y, Goto Y, Umaoka Y, Shiotani M, Nakayanishi Y, Mori. Culture of human embryos in alpha modification of Eagle's medium under low oxygen tension and low illumination. Fertil Steril 1994; 62:1022.

284. Bedford JM, Dobrenis A. Light exposure of oocytes and pregnancy rates after their transfer in the rabbit. J Reprod Fertil 1989; 85:477.
285. Paria BC, Dey SK. Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. Proc Natl Acad Sci USA 1990; 87:4756.
286. Lane M, Gardner DK. Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. Hum Reprod 1992; 7:558.
287. Salahuddin S, Ookutsu S, Goto K, Nakanishi Y, Nagata Y. Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of in-vitro fertilized mouse embryos. Hum Reprod 1995; 10:2382.
288. Gardner DK, Lane MW, Lane M. Development of the inner cell mass in mouse blastocyst is stimulated by reducing the embryo: incubation volume ratio. Hum Reprod 1997; 12:Abstract Book 1, p-132.
289. Ahern TJ, Gardner DK. Culturing bovine embryos in groups stimulates blastocyst development and cell allocation to the inner cell mass. Theriogenology 1998; 49:194.
290. Gardner DK. Improving embryo culture and enhancing pregnancy rate. In: Shoham Z, Howles C, Jacobs H, eds. Female Infertility Therapy: Current Practice. London: Martin Dunitz, 1998:283.
291. Miller KF, Goldberg JM, Collins RL. Covering embryo cultures with mineral oil alters embryo growth by acting as a sink for an embryotoxic substance. J Assist Reprod Genet 1994; 11:342.
292. Erbach GT, Bhatnagar P, Baltz JM, Biggers JD. Zinc is a possible toxic contaminant of silicone oil in microdrop cultures of preimplantation mouse embryos. Hum Reprod 1995; 10:3248.
293. Croxatto HB, Ortiz ME, Diaz S, Hess R, Balmaceda J, Croxatto HD. Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. Am J Obstet Gynecol 1978; 132:629.
294. Marek D, Langley M, Gardner DK, Confer N, Doody KM, Doody KJ. Introduction of blastocyst culture and transfer for all patients in an in vitro fertilization program. Fertil Steril 1999; 72:1035.
295. Wilson M, Hartke K, Kiehl M, Rodgers J, Brabec C, Lyles R. Integration of blastocyst transfer for all patients. Fertil Steril 2002; 77:693.
296. van Os HC, Alberda AT, Janssen-Caspers HA, Leerentveld RA, Scholtes MC, Zeilmaker GH. The influence of the interval between in vitro fertilization and embryo transfer and some other variables on treatment outcome. Fertil Steril 1989; 51:360.
297. Buster JE, Bustillo M, Rodi IA, et al. Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage. Am J Obstet Gynecol 1985; 153:211.
298. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 1988; 332:459.
299. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schonauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. Hum Reprod 2001; 16:1115.

300. Veeck LL. Does the developmental stage at freeze impact on clinical results post-thaw? Reprod Biomed Online 2003; 6:367.
301. Veeck LL, Bodines R, Clarke RN, et al. High pregnancy rates can be achieved after freezing and thawing human blastocysts. Fertil Steril 2004; 82:1418.
302. Anderson AR, Weikert ML, Crain JL. Determining the most optimal stage for embryo cryopreservation. Reprod Biomed Online 2004; 8:207.
303. Papanikolaou V. Early pregnancy loss is significantly higher after day 3 single embryo transfer that after day 5 single blastocyst transfer in GnRH antagonist stimulated IVF cycles. RBM Online 2006; 12:60.
304. Gardner DK, Balaban B. Choosing between day 3 and day 5 embryo transfers. Clin Obstet Gynecol 2006; 49:85.
305. Papanikolaou EG, et al. Live birth rate is significantly higher after blastocyst transfer than after cleavage-stage embryo transfer when at least four embryos are available on day 3 of embryo culture. A randomized prospective study. Hum Reprod 2005; 20:3198.
306. Papanikolaou EG, D'haeseleer E, Verheyen G, et al. In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. New England Journal of Medicine 2006; 354:1139.
307. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 2000; 73:1155.
308. McKenzie LJ, Pangas SA, Carson SA, et al. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Hum Reprod 2004; 19:2869.
309. Preis KA, Seidel G Jr, Gardner DK. Metabolic markers of developmental competence for in vitro-matured mouse oocytes. Reproduction 2005; 130:475.
310. Weiss TJ, Warnes GM, Gardner DK. Mouse embryos and quality control in human IVF. Reprod Fertil Dev 1992; 4:105.
311. Gerrity M. Mouse embryo culture bioassay. In: Wolf DP, ed. In Vitro Fertilization and Embryo Transfer. New York and London: Plenum Publishers, 1988:57.
312. Fukuda A, Noda Y, Tsukui S, Matsumoto H, Yano J, Mori T. Influence of water quality on in vitro fertilization and embryo development for the mouse. J In Vitro Fert Embryo Transf 1987; 4:40.
313. George MA, Braude PR, Johnson MH, Sweetnam DG. Quality control in the IVF laboratory: in-vitro and in-vivo development of mouse embryos is unaffected by the quality of water used in culture media. Hum Reprod 1989; 4:826.
314. Silverman IH, Cook CL, Sanfilippo JS, Yussman MA, Schultz GS, Hilton FH. Ham's F-10 constituted with tap water supports mouse conceptus development in vitro. J In Vitro Fert Embryo Transf 1987; 4:185.
315. Davidson A, Vermesh M, Lobo RA, Paulson RJ. Mouse embryo culture as quality control for human in vitro fertilization: the one-cell versus the two-cell model. Fertil Steril 1988; 49:516.
316. Fleming TP, Pratt HP, Braude PR. The use of mouse preimplantation embryos for quality control of culture reagents in human in vitro fertilization programs: a cautionary note. Fertil Steril 1987; 47:858.

317. Montoro L, Subias E, Young P, Baccaro M, Swanson J, Sueldo C. Detection of endotoxin in human in vitro fertilization by the zona-free mouse embryo assay. Fertil Steril 1990; 54:109.
318. Rinehart JS, Bavister BD, Gerrity M. Quality control in the in vitro fertilization laboratory: comparison of bioassay systems for water quality. J In Vitro Fert Embryo Transf 1988; 5:335.
319. Bavister BD, Andrews JC. A rapid sperm motility bioassay procedure for quality-control testing of water and culture media. J In Vitro Fert Embryo Transf 1988; 5:67.
320. Stewart-Savage J, Bavister BD. Deterioration of stored culture media as monitored by a sperm motility bioassay. J In Vitro Fert Embryo Transf 1988; 5:76.
321. Reed ML, Petters RM. An evaluation of hamster, rat, and mouse sperm-cell motility in media formulated with water of different qualities. J In Vitro Fert Embryo Transf 1991; 8:41.

14

Micromanipulation as a Clinical Tool

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INTRODUCTION

Micromanipulation involves a well-integrated set of technologies in assisted reproductive technology (ART). Its applications are diagnostic as well as therapeutic, and it is practiced in mature gametes and all stages of preimplantation embryos. It is used in biopsy for preimplantation genetic diagnosis (PGD), intra-cytoplasmic sperm injection (ICSI), assisted hatching, and in other more controversial areas such as egg freezing via zygote reconstitution, cryopreservation of isolated testicular spermatozoa, and cytoplasmic or mitochondrial transfer for reversal of cytoplasmic and potentially nuclear incompetence (1–6). In this context, it becomes increasingly difficult to discuss micromanipulation as a separate subject. In fact, the need for a separate assessment appears almost artificial.

When different fields merge in science, exciting developments can be expected. This has occurred numerous times in ART, first in the integration of biochemistry and reproductive endocrinology, and later when cryobiology and applied genetics emerged as tools to improve efficiency and safety. No reproductive specialist could have predicted that the field of experimental micromanipulation would have such an enormous impact on assisted reproduction less than two decades after the first relatively simple but elegant applications appeared (1–3). Since then, hundreds of thousands of

babies have been born worldwide from micromanipulative methods aimed at alleviating male infertility and enhancing implantation and the exclusion of chromosomal and single gene disorders. Some laboratories now have three or more complete stations for micromanipulation. Rather than having some embryologists sub-specialize in the area of micromanipulation, the practice in some laboratories, most embryologists now aim to become proficient in one or more micromanipulation techniques.

The main emphasis here will not be already integrated procedures such as ICSI and in part embryo and polar body biopsy, which are covered in depth by other chapters of this book, but on other innovative techniques. The different procedures and concepts will be discussed in two sections; the first will focus on gamete micromanipulation and the second will deal with the manipulation of embryos. It is also important to note that the topics discussed in this chapter are often considered controversial or unproven and are seen by some as hazardous to clinical care and even the human species at large. No review would be complete if it does not refer to the alternative opinion. A recent 2004 opinion by Cummins gives a very different perspective of some of the technologies described below (7).

GAMETE MICROMANIPULATION

Cryopreservation of Spermatozoa Under Zonae

Men who are azoospermic can be successfully treated through surgical isolation of spermatozoa from their testicles or reproductive tract (8–10). Repeated surgical procedures, however, may not only be costly but invasive, especially in the case of testicular sperm extraction (11). Repetition can, in some cases, be avoided by normal cryopreservation of spermatozoa, but only when sufficient numbers of functional cells are isolated (12,13) Freezing methods are now available that can freeze and recover very few or even single spermatozoa, and avoid the need for repeated surgical sperm extraction (4,14,15). Single sperm can be frozen by insertion of spermatozoa into animal or human evacuated zonae pellucidae or through variations of this method such as freezing in cryoloops.

Recovery rates of spermatozoa frozen and thawed in evacuated human or animal zonae are high, with motility recovery rates in excess of 75% (4,15). In standard freezing protocols, centrifugation is essential. But in this protocol, washing can be accomplished by individually pipetting and removing the cryoprotectant.

In addition to reducing the need for repeated sperm retrievals and perhaps donor sperm, this approach has the advantage of avoiding the uncertain outcome of surgical extraction by freezing spermatozoa and retrieving eggs at different times (4,16). The time-consuming search for spermatozoa can be conducted independent of an egg retrieval.

Details of Procedure and Choice of Technical Details

Micromanipulation can be performed using polyvinyl pyrrolidone (PVP) as a tool to slow the insertion of spermatozoa into the zonae and to withdraw spermatozoa from the zonae after thawing. Two different solutions of PVP are recommended: (i) an 8 to 10% solution for sperm capture and insertion into empty zonae (this is produced by a number of manufacturers with varying results) and (ii) a 10 to 12% solution for sperm recovery from the thawed zonae. The ICSI procedures using thawed spermatozoa should be performed at 37°C, but all other micromanipulations can be performed at room temperature in order to reduce sperm velocity and perhaps prolong survival. The microtools needed for zona opening, avoidance of zona collapse, cell extraction, and ICSI have been described (3,4).

The pilot experiments were conducted using spermatozoa from surgical retrievals and spermatozoa not cryopreserved from men with normal semen analysis to test the fertilizing ability of donated research oocytes by ICSI. Human-evacuated zonae can be obtained from multiple sources: immature eggs, unfertilized ICSI eggs, and abnormal embryos that were not exposed to sperm suspensions.

It was shown that two small incisions in the zonae improved extraction of the egg ooplasm and insertion of the sperm cells into the evacuated zonae by preventing the collapse of the zona during suction and excessive inflation. With gained experience, a single-hole technique may be effective as well. At first, holes were made chemically in some pre-fertilization zonae by releasing acidified Tyrode's solution from a 10 μm open microneedle. However, progressively motile spermatozoa escaped, resulting in poor recovery rates. This can be avoided by cutting a hole in the zona mechanically using partial zona dissection with a spear-shaped closed microneedle. Alternatively, one can use a laser-mediated opening of the zona pellucida, but the efficiency of this needs to be shown in clinical trials (17).

Cytoplasm can be extracted using a larger micropipette, connected in turn to a suction device. The zona is positioned so that one of the two incisions is at the 3 o'clock position. The beveled microtool is inserted through the aperture using the sharp edge on the lower end. The tool is moved through the oolemma, and the cytoplasm is fully aspirated until the zona is empty. The pipette is occasionally emptied outside the zonae as more medium is sucked up to remove any sticky cytoplasm from the pipette tip. Mouse and hamster zonae as well as human eggs and embryos can also be prepared in the same fashion.

Spermatozoa can be released into the 10% PVP solution prior to insertion into empty zonae. They are individually taken from small 2- to 5-μL droplets of sperm suspension using an ICSI microtool. Spermatozoa can be injected into the empty zona while motile or can be immobilized before

injection and freezing (17). In the latter case, the spermatozoa remain viable at a high frequency, but fertilization and pregnancy have not yet been demonstrated. Considering the absence of clinical data, motile spermatozoa are recommended for this purpose. The use of human zonae, while appropriate for creating strict xeno-free conditions, is problematic as spermatozoa attach to the inside of the zona pellucida and may immobilize before freezing. Recently, we developed and tested a combinatorially derived ligand pieczenik peptide sequence 2 from an artificial target that specifically binds to human zonae pellucidae (18). When injected inside evacuated human zonae, this ligand efficiently prevented sperm attachment by possibly interfering with sperm/zona pellucida (ZP3) interaction. Whether this ligand could be used for the purpose of freezing spermatozoa in evacuated zonae and maintaining high motility requires further evaluation.

Injected zonae can be frozen in a simple 8% glycerol solution using a phosphate-buffered solution supplemented with 3% human serum albumin. Alternatively, one can use buffer containing TES (N-tris[hydroxymethy]-methyl-2-aminoethanesufonic acid) and Tris (Tris[hydroxymethyl]amino-methane) yolk buffer, but the recovery rate is not superior compared to earlier studies (16). The zonae can be frozen singly in standard plastic straws between two small air bubbles to indicate their position. One end of the straw is closed using sealant polyvinyl alcohol powder, whereas the other end is heat sealed. The freezing procedure is based on a simple standard semen cryopreservation protocol (4).

Thawed straws can be inserted into a medium droplet as the cryopreservation medium containing the zona slowly releases. The zona is washed to remove cryoprotectant and moved into an ICSI dish containing droplets of N-2-Hydroxyethylpiperazine-N′-2-Ethanesulfonic acid (HEPES) buffered medium and a central droplet of 10 to 12% PVP in supplemented intracellular solution. Some sperm cells may show considerable motility within the zona. The zona is positioned using the holding pipette and a PVP-filled ICSI microtool so one incision and a sperm cell line up. This allows for the penetration of the ICSI needle and aspiration of the sperm cell (mechanical recovery method). Minimum suction is used for this process. These cells can be aspirated by positioning the needle at the contralateral side and applying suction when the sperm cell passes the needle aperture. All spermatozoa are removed and released gently in the PVP solution. Any still motile can then be immobilized for ICSI. The mechanical sperm recovery method is preferred, and other methods involving zona digestion have proved less successful. Recovered sperm are washed and immediately injected into eggs; thaws should be planned accordingly.

Zonae are rarely lost with this method as they are still heavy enough to drop to the bottom of a dish when the straw's contents are released. The number of sperm lost through holes after thawing ranges from 2 to 30% and is dependent on the technique (4,15,16). Spermatozoa are not lost before freezing

through the narrow mechanical PZD incisions, but some may be lost when using acidified Tyrode's solution or laser opening. Loss after thawing through narrow incisions may also occur because of inadvertent excess suction applied through the holding pipette. This can be avoided by visualizing both holes prior to micromanipulation and sperm recovery. The rate of sperm loss through the incisions diminishes markedly with increased operator experience.

Motility Recovery and General Efficiency

Motility recovery rates are defined as the percentage of motile cells seen, and vary between 73 and 100%. Rates of recovery over 80% are found in experiments involving rodent zonae and human zonae that are embryonic in origin, or spermatozoa that are immobilized prior to freezing. Some cells that are motile without marked velocity become progressively more motile after aspiration and exposure to medium. Some spermatozoa inserted into human zonae may become caught up in cytoplasmic remnants or crevices inside the glycoprotein matrix, but this occurs less frequently when animal zonae are used. Aggregation of spermatozoa also appears to inhibit individual motility, but is avoided when three or less spermatozoa are inserted per zona. The use of only one to three sperm cells per zona pellucida appears optimal, yet insertion of up to 15 spermatozoa has been reported (16).

General Considerations of Single Sperm Freezing

Although single sperm freezing is performed with spermatozoa from men with extreme oligospermia, average motility recovery rates are considerably higher than those generally reported for moderately abnormal semen and are comparable to results of donor semen or moderately oligospermic patients treated with a combination of cryoseeds and dithiothreitol (DTT) (19–21). The use of the zona pellucida or perhaps cryoloops as a vehicle avoids the known loss in motility associated with post-thaw dilution and sperm washing seen in frozen donor semen. It is possible that the presence of multiple sperm in a small volume exerts an internally deleterious effect during freezing, thawing, and centrifugation, which may be avoided by freezing sperm singly or in small groups. This method makes it feasible to perform surgical extractions independently from the time and place of egg retrieval.

Another distinct advantage is that animal zonae, such as those from mice and hamsters, can be used for storage. This application has been rejected by some because of the desire to culture under xeno-free conditions. Although it is immunologically unlikely that there will be any cross-species hazards or reactive compounds, it is possible that compounds may adhere to the sperm cell and become incorporated in the oocyte. Whether these have any metabolic effects on the embryo has yet to be clarified. Nevertheless, the absence of reverse transcriptase appears evident, thereby rigorously reducing the possibility of any process resembling transgenesis.

The use of combinatorially derived ligands as described above for inhibiting sperm–egg binding in the homologous application may be preferred, but should be tested in pre-clinical models first.

Clinical Application

The experimental procedure was first tested in an azoospermic patient who required testicular biopsy and had scant motile cells (22). Extensive searching for sperm yielded enough spermatozoa for ICSI. Other motile spermatozoa ($n = 23$) were isolated and trapped into two evacuated zonae derived from the patient's immature eggs. The patient did not become pregnant and the couple returned for another ICSI attempt, but this time without sperm retrieval by testicular biopsy. One zona with 12 trapped spermatozoa was thawed first, washed, and prepared for sperm extraction. All but one sperm could be recovered. Ten were motile, and five were injected into the patient's mature eggs four hours after egg retrieval. The patient delivered healthy twins.

A total of 104 procedures have been performed, and 52 of them returned for transfer. Of these, 49 had transfers and 26 became clinically pregnant. The implantation rate was nearly 25%. There were three miscarriages and two biochemical pregnancies. Although a small data set, the work is encouraging but awaits confirmation by other teams.

Ooplasmic Transplantation

Transplantation of ooplasm for clinical application involves a set of experimental techniques designed to address oocyte-specific deficits in a defined and limited group of infertile patients who previously failed ART (5,23). The first clinical application was based on oocyte donation, although the use of homologous ooplasm or mitochondria from the patient's cumulus cells has also been suggested (24–26). The latter proposal was first made by Tzeng's team at Taipei Medical University in Taiwan and this has led to the birth of more than 20 babies. There are a number of ways to transplant ooplasm, but the one initially used was a minor modification of the standard clinical ICSI technique. A small portion of ooplasm was transferred from a donor to the patient's oocytes. Other groups have reported on the experimental application of modified versions of ooplasmic transfer, including the use of cryopreserved donor oocytes, polyspermic zygotes, and cumulus cell mitochondrial suspensions as the source of the donor infusion (24,27,28). In the work performed from 1997 to 2001 at Saint Barnabas Medical Center's Institute for Reproductive Medicine and Science, two instances of abnormal karyotypes were reported following the application of ooplasmic transfer (both 45, XO). One resulted in an early spontaneous miscarriage and the second in an elective reduction at 15 weeks following an abnormal ultrasonography (29). A single case of pervasive developmental disorder (a spectrum of autism-related diagnoses which has an incidence of one in 250 children) was also diagnosed at 18 months in a ooplasmic

transfer infant, a boy from a mixed sex twin. Other centers applying ooplasmic transplantation have not reported any potential side effects. This may be because there were no side effects or the studies were incomplete. Due to the very small sample size, any direct connection between the abnormalities and the technique itself has yet to be established (30).

In 2001, we voluntarily agreed to cease clinical application of ooplasmic transfer pending the application and review of an investigative new drug (IND) protocol with the U.S. Food and Drug administration. The pre-IND process was concluded in 2003, but we did not pursue the application further for non-clinical reasons. The procedure was not prohibited as Schultz and Williams (31) and others have suggested. It is currently unknown whether other groups have applied for a permit.

The clinical application and science of ooplasmic transplantation have been extensively criticized by ethicists and scientists (7,31–33). Some careless articles of the lay press, fueled by some scientists, have likened the technique to gene transfer and described a detrimental artificial scenario likely to change the genetics of mankind. Several recent publications have presented viewpoints based on a poor understanding of the issues. The debate should be based on a factual understanding of the procedure and the clinical and scientific realities involved. The technique's underlying experimental nature must be stressed and the many scientific and clinical "unknowns" that surround it must be presented.

Ooplasmic Transfer Procedures

Preliminary clinical investigations applying the technique to a defined group of patients with "normal" ovarian reserve who exhibit consistent developmental problems and implantation failure have been published (29,34). The final 27 couples treated had exhibited a record of failure in over 95 prior assisted reproduction treatment cycles. Unlike what some critics have suggested, this is an unavoidable confounding factor in attempting to design and conduct true controlled trials (33,35). In this case, the "control" treatment is already consistently known to result in failure and the cause is not unknown since embryo morphology was clearly marginal in all these cases. The 43% pregnancy rate and the delivery of 17 babies following ooplasmic transfer was characterized solely on this prior failure criteria. The issue of controlled trials is obviously complex, and to suggest that this is a simple deficit that we have failed to address is incorrect. There is a considerable ethical issue in forcing patients to engage in treatment modalities that offer them no hope of success for the sake of these trials. In a subsequent study, patients with diminished ovarian reserve were treated unsuccessfully with cytoplasmic transfer (36). It is known that in these patients most eggs are aneuploid, a condition that is irreversible at the MII stage (37).

Ooplasmic transfer was conceived as a simple but critical extension of the standard egg donation protocol that is a clinical option available for patients. It theoretically provides for a beneficial donor egg while

maintaining the patient's genetic contribution. The technique is suitable for eggs "having a normal nuclear genome, but ooplasm that is abnormal or deficient due to maternally mediated factors" (38). Recently, several authors have incorrectly represented our publications as suggesting that ooplasmic transfer is based on a correction of deficits in mitochondrial function or ATP content (32,33). One publication goes so far as to raise this purported ATP deficit as a "straw man" argument and, in rejecting it, raises the question that ooplasmic transfer might not be "biologically plausible." We have clearly stated in our publications that there are a multitude of causative factors potentially underlying ooplasmic-related developmental deficits. Energy metabolism is certainly one of these factors. However, the entire concept of ooplasmic transfer is to infuse the potentially compromised patient oocyte with a whole source of healthy donor ooplasm. We have never suggested that a manipulation of ooplasmic ATP or any specific factor underlies any positive effect of ooplasmic transfer.

Others have suggested that mitochondria transfer from isolated cumulus cells would be a safer alternative, but we reject the direct comparison based on the arguments outlined here (24,25). Mitochondria transfer has been considered sub-optimal compared to ooplasmic transfer in one artificial mouse model (39). It is likely that a subset of infertile patients exhibit reproductive dysfunction derived from oocyte-related deficits and that replacing compromised oocytes with donor substitutes is not only "biologically plausible" but also a technique compatible with early development and pregnancy (40).

Mitochondrial Issues

An area of controversy concerns the transfer and persistence of donor mitochondria following ooplasmic transfer. The transfer of heterologous mitochondria was not detectable in the first ooplasmic transfer cases, and therefore initial reports and discussions reflected this (38). The oosplasmic transfer protocol has included an analysis of mitochondrial DNA and, in subsequent treatment cycles, donor mitochondrial DNA was identified in pre- and postnatal samples derived from ooplasmic transfer offspring. To date, donor mitochondrial DNA has been positively identified in three of the 13 tested ooplasmic transfer babies (34). This suggests a heteroplasmic condition with two populations of mitochondria (donor and recipient) present.

However, current scientific evidence does not support the concept that ooplasmic transfer-related heteroplasmy constitutes a potentially deleterious condition. The only heteroplasmy that has been observed in a small subset of ooplasmic transfer patients is in the form of benign polymorphisms in the non-coding hyper-variable region of the otherwise highly conserved mitochondrial genome (34). This form of heteroplasmy is now known to be a common phenomenon in the normal human population and may not have an association with mitochondrial disease or dysfunction (41). Indeed, this form of benign heteroplasmy may have little significance. Many mammalian species are routinely heteroplasmic in the replication control region, and this

heteroplasmy may be conserved and transmitted from generation to generation (42,43). This benign form of heteroplasmy is different from that associated with deleterious coding region mutations related to "aging and in inherited mitochondrial disease" as incorrectly suggested in one critical opinion (33). Selection of young and healthy oocyte donors providing gametes for ooplasmic transfer is based on the same criteria as standard oocyte donation. Ooplasmic transfer patients have no unique risk of the transmission of such rare deleterious mitochondrial DNA mutations as whole oocyte donation is the only other clinical option available for them. Suggestions that ooplasmic transfer donors should be uniquely screened for mitochondrial mutations are more logically directed at oocyte donation where 100% of the mitochondrial genome is derived from the donor (32). No incidence of mitochondrial disease transmission has been reported over 10 of 1000 of oocyte donation cycles, although it is expected that 1 of 8000 children will develop mitochondrial disease de novo.

A substantial body of animal research has been concerned with the manipulative creation of heteroplasmy in mice and large animals [reviewed by Malter and Cohen (29)]. Although the opinion piece by St John (32) mentions this research, it fails to point out the underlying fact that much of this research is based on the efficient generation of hundreds of healthy heteroplasmic animals that have been produced through cytoplasmic transfer and maintained over 15 generations with no obvious developmental or physiological problems. From a genetic standpoint, many of these experiments are also based on a much more drastic heteroplasmic scenario as the mixed mitochondrial populations are essentially derived from two ancestrally different species. While one should not place tremendous confidence in modeling a complex phenomenon through research animals, in this case such research has demonstrated that a much more extreme heteroplasmic condition than would be possible through clinical ooplasmic transfer is compatible with normal mammalian development. Other basic research suggests considerable flexibility in nuclear/mitochondrial interaction, particularly among primates. In the type of cellular hybrid experiments also discussed by St John (32), chimp and gorilla mitochondria could readily replace human mitochondria (a cross-genus "mismatch"), and create fully functional cells with human nuclear genomes and non-human primate mitochondria exhibiting unremarkable mitochondrial protein synthesis and function (44). Although the specific nature of the heteroplasmy observed in a small fraction of ooplasmic transfer offspring is not fully understood, an honest review of current research in this area does not suggest a potential for negative developmental or physiological outcomes. This obviously does not mean that future patients should not be informed of the uncertainties that remain.

Epigenetic Aspects and Animal Models

Ooplasmic transfer, by design, generates a recipient oocyte that contains donor-derived components such as proteins, messenger RNAs, mitochondria,

and other cytoplasmic constituents. In theory, this infusion of healthy donor components can have a positive effect on important ooplasmic functions during early development. However, a recent opinion piece by Hawes et al. suggests a potential for adverse developmental outcomes resulting from the simple creation of a mixed ooplasmic state (35). This argument is based on abnormal developmental syndromes that have been identified in unique inbred mouse strains [reviewed by Malter and Cohen (29)]. These syndromes result from genetic incompatibilities between inbred strains apparently manifested via unique epigenetic events in the cytoplasm of their oocytes and early embryos.

Studies with these inbred strains have been critical to understanding the early cytoplasmic genome modification events that create the mature functional embryonic genome (29). The epigenetic processing that occurs during this period is necessary for proper development. This supports the concept that positive developmental effects could be obtained by a moderate infusion of healthy ooplasm, although other outcomes, including negative effects, are plausible as well. The scenarios and experimental manipulations involved with the manifestation of these aberrant developmental outcomes are also essentially compatible with normal development in the mouse (and other species) outside of these experiments. In our own research, similar manipulation to the early cytoplasm of F1 hybrid mouse embryos resulted in a significant improvement in certain developmental parameters compared to non-manipulated controls [Levron et al. (6)]. It has been suggested that similar alleleic combinations for these unique incompatible murine gene products could be present in the human, yet there is no proof of this.

The aberrant developmental outcomes observed in the inbred mouse strains are unique epigenetic anomalies with clear genetic causes (45,46). These specific deleterious anomalies are unknown outside of the unique inbred combinations used and any other animal model system. Inbred mice are genetically anomalous strains considered to be homozygous at all loci. They manifest a great variety of adverse developmental and physiological conditions including reduced fertility (29). Even between related inbred strains, drastic differences in morphology, physiology, and behavior make drawing cross-strain conclusions questionable. Such strains do not constitute valid models for complex processes (particularly those related to fertility and development) in other mammalian species. Furthermore, many differences between the developmental processes in humans and other mammalian species have been well characterized, including a recent finding which demonstrates clear differences in the methylation-based imprinting system (47). In fact, the authors of this study go so far as to suggest that concerns about the aberrant epigenetic-processing (and resulting developmental effects) observed after in vitro manipulation in other mammalian species and model systems may simply not apply to the human.

Therefore, to suggest, based on artefacts and tenuous evidence, that deleterious epigenetic combinations could likely be created by clinical ooplasmic transfer—where the small volume of infused ooplasm is derived from another highly outbred, healthy, and fertile human donor—seems speculative at best. Unfortunately, this is an example of a growing trend in the use of questionable animal model systems to judge the efficacy and safety of human procedures.

Despite these points, considerable effort and financial support from the governments are routinely expended on animal-based studies involving highly artificial conditions that assert some kind of relevance to the human. This type of research is now frequently being used to attack and question clinical procedures. Procedures such as in vitro fertilization (IVF) and ICSI have long established histories of safety and efficacy in humans proven over 10 of 1000 of successful treatment cycles. Complex medical follow-up of IVF outcomes is performed by obtaining data from comparable age groups. To discount direct data based on the minor results of contrived animal model systems with tenuous physical, physiological, and developmental connections to the human is questionable science.

Obviously, assisted reproduction is not safe beyond any doubt. Conclusive data has been published regarding reduced birth weight of IVF singletons and congenital malformation rates that suggest there may be inherent problems associated with treating infertile patients (48,49). These problems may be associated with follicular stimulation, in vitro manipulation, or an altered reproductive condition. Some extensive follow-up studies have corrected prior (and current) suggestions that pre- and post-natal development may be compromised by procedures (50). Control groups of the general population are not really appropriate because they do not suffer from the same condition(s).

One recent study distinguishes between the inherent etiology of the study population (the infertile and possibly prenatal and peri-natal conditions that are associated with this) and consequences of hormonal alteration of the ovary and in vitro culture (51).

How Should Human Infertility Treatment Advance?

The first experimental clinical application of a new technique in reproductive medicine always raises the question of the possible negative effects on the patient, offspring, and subsequent generations; this is certainly a sober and critical issue in assessing the potential risks and benefits of the application. The question has been raised throughout the history of human infertility treatment, occasionally in a panic-stricken fashion that often prevents progress in this area. The current state-of-the-art in the field has arisen through a complex process involving patients, physicians, scientists, ethicists and, with increasing frequency, government and social entities. It has slowly advanced through research, and the development and clinical application of

experimental techniques like ooplasmic donation that address deficits in our capabilities derived from needs in the patient population. Patients have acknowledged and accepted risks to future generations on their behalf, although this remains a valid ethical question.

We feel strongly that ooplasmic transfer was developed and experimentally applied in a reasonable and responsible fashion. Others beg to differ [for full opposing review see Cummins (7)]. The decision to proceed with experimental clinical trials of ooplasmic transfer was based on a careful review of mid-1990s scientific knowledge in this area (including animal models, mitochondrial issues, and epigenetic aspects) as well as considerable direct experience with successfully incorporating advanced manipulative techniques into the human clinical environment. Suggestions that experimental application of ooplasmic transfer in humans proceeded in the absence of prior research in this area are incorrect as any unbiased review of the pertinent literature will demonstrate (29).

Appropriate ooplasmic transfer patients ($n = 33$) were selected from a much larger group of potential patients (several thousand) who expressed a strong interest in participating in such a trial. These patients were carefully informed of all potential negative aspects of the technique including physical, developmental, mitochondrial, and epigenetic aspects. They participated in an informed consent process that was supervised by our hospital internal review board and constantly updated to include all pertinent information on ooplasmic transfer results. This included the chromosomal abnormalities observed, the incidence of heteroplasmy, and criticism from bio-ethicists and basic scientists. Internal review board supervision of informed consent is an ethical and responsible process that works well to protect patients and allow for clinical advancement. Patient follow-up is also a critical component of an experimental trial.

POST-FERTILIZATION

Assisted Hatching

General Considerations

The premise of assisted hatching is based on the now 17-year-old hypothesis that modification of the human zona pellucida, either by its elimination, by drilling a hole through it, by thinning it, or by altering its stability, will promote hatching or implantation of embryos that are otherwise unable to escape intact from the zona pellucida (2). As this argument is based on data from eggs obtained from follicular stimulation and in vitro observations involving IVF, none of the work suggests that there is a true disease-specific condition causing infertility because of this. The hypothesis reflects on the in vitro fertilized egg only, and as such the technology has become a conundrum, with only a minority of "believers." It is assumed that assisted

hatching is not widely practiced based on the fact that there are only about 250 scientific papers regarding this topic in the literature, in sharp contrast with, for instance, the ICSI literature. Whereas there is a broad consensus about efficacy regarding the latter, this is clearly not the case with assisted hatching. A large proportion of studies have evaluated minor changes in the technical protocol without emphasis on clinical efficacy. Five meta-analysis studies have been published to date about assisted hatching, but three were biannual updates from the same team (52–56). The consensus in these studies is that assisted hatching improves outcomes in poor prognosis patients, particularly in the case of maternal aging, not all that different from our conclusions reported in 1993 after conducting four randomized trials (57). Proof of efficacy was only attainable when patients were selected based on maternal age or prior failed attempt. The research team that has published three consecutive meta-analysis studies fails to explain the difficulty when randomizing unselected or partially selected populations. In our work with over 10,000 patients, the effect of assisted hatching is not noticeable because we are unable to select against suitable control patients, so once one has a track record from randomized studies, a data dilution effect is likely to result. When controlled studies are compared, there are no good ways to compensate for study design differences such as those caused by age cut-off, opening, thinning, and subtle variation in methodologies and number of attempts. Hence, the comparisons become blurred, similar to attempting to evaluate the parameters of a single large IVF database with specific groups represented in both experimental and control arms. In addition, embryo culture technology and follicular stimulation, which are both the most likely factors affecting embryonic health and associated zona changes, have improved spectacularly during the past ten years. This has possibly reduced the need for assisted hatching in sub-groups of patients, yet the exact contribution of this change in technology on hatching behavior needs to be determined. One factor that appears missing in all review papers of assisted hatching is the lack of understanding of nuances of techniques within each class of technologies used. It does not matter whether one uses laser, acidified Tyrode's solution, enzymes, mechanical opening, or some other derivative technology—there are only few appropriate ways of doing each technique, but simply no guidelines for this in the literature. Indeed, this does not only apply to assisted hatching but also to any medical technology. Most practitioners realize that there are ways of doing poor IVF and good IVF. Likewise, there is also good assisted hatching technique and poor assisted hatching technique. Meta-analysis evaluations are not able to weigh these important aspects. Indeed, nor can this reviewer provide a more exact evaluation of the problem, but nevertheless, as the one who introduced assisted hatching, I would like to take this opportunity to share some observations with the reader without evaluating each of the assisted hatching papers one by one. Other reviews have done this already and the more

recent ones will illustrate the difficulty in determining the efficacy of assisted hatching and the lack of consensus among practitioners (58,59).

The most common technology used is that of opening the zona pellucida with acidified Tyrode's solution (57). The pH of zona breaching is about 2.3; below this the zona will disappear as a coherent structure, and above it the zona will remain intact. In this respect it is important to consider the buffer solution that the embryos are kept in during the procedure. Some buffers, such as HEPES, are excellent in locally maintaining pH and have high buffering capacity. These solutions are more forgiving than bicarbonate buffered systems which have reduced buffering capacity. Another consideration is that technologists, while aware of the aggressiveness of acidified solution and therefore often like to perform the procedure gently and carefully, are not necessarily considering the physics of the system. Also, a hierarchy of teaching from developer to practitioner, as was the case with ICSI, is largely absent as embryologists have interpreted zona opening as a very simple procedure and have applied the technique directly by reading the original publications without communicating with the experienced groups. While the first descriptions had some depth, it has become apparent over time that subtle aspects were not described in detail. Hence, there have been a number of interpretations of the procedure and perceptions of this being an easy technique have largely prevailed. An example of this is that many embryologists will not deposit the acidified solution directly on the zona pellucida while keeping the microneedle pressed on the zona pellucida. This to many embryologists would seem aggressive, yet it is the only way to reduce release of very limited amounts of acidified solution. While the zona is dissolving, the microneedle should be moved into the thinning area. Keeping the microneedle at distance and releasing acidified solution will cause a broad stream of acidified medium insufficiently lowering the pH below 2.3, the result being that larger quantities of acidified solution are being released because of perceived carefulness. This is likely to affect cells adjacent to the manipulated area. Similar considerations can be applied to the use of laser. The lack of referring to possibly detrimental effects in this respect in the original clinical applications is worrisome.

Less than half of all the embryos created after assisted reproduction appear genetically normal (60), but implantation rates are generally lower than that, indicating that a number of other factors must be involved. Assisted hatching promotes earlier implantation and may therefore elevate the chance of implantation by optimizing the implantation window (61). There is additional proof that superficially thinning of the zona pellucida is advantageous in certain patients (62).

Complete removal of the zona pellucida prior to compaction may lead to loss of cells due to the absence of structural junctions. Only a proportion of human expanded blastocysts growing in vitro will hatch and this frequency may be dependent on the quality of the culture system. The incidence of

hatching in vitro is enhanced by zona opening, at least in a proportion of in vitro studies. Assisted hatching can potentially be applied to any embryo, but its application by clinics has been slow or it has been abandoned in the absence of consistent results because of technical variations and subtle changes to the originally published protocols. Common problems identified by us have been (i) the lack of immunosuppression (63) in patients whose embryos are zona-drilled, (ii) the excessive use of acidified Tyrode's solution by keeping the micro-pipette more than 5 μm away from the zona pellucida, thereby decreasing the pH of a greater area than is needed for zona piercing as described above, (iii) the use of hand-controlled suction devices for release of acidified solution, which does not allow controlled release due to insufficient visualization of the fluid, (iv) the creation of holes smaller than 10 μm which may trap the embryos during hatching, (v) the creation of holes larger than 25 μm which may lead to cell loss during or after embryo replacement, and (vi) the inability to change the transcervical embryo transfer method in such a way that excess zona pressure is avoided during replacement.

At least five methods have been used with varying amounts of success: (i) partial zona dissection on day-two, three, or day 5, (ii) zona drilling with acidified Tyrode's solution on day-three, (iii) laser-assisted drilling on day-three using an infrared non-contact laser, (iv) zona drilling with either method at the blastocyst stage and (v) piezo-mediated drilling was also achieved in animal and human models and might in future be considered an alternative for clinical application (64), yet results in our laboratory have shown that excessive use of piezo devices is detrimental for embryo development in the mouse.

We abandoned the use of the first method of partial zona dissection as it may produce gaps which are too small, possibly resulting in cell separation during the escape from the zona pellucida (65). The creation of a second perpendicular gap may be beneficial (66). Larger mechanical openings may be helpful and indeed have been studied to some extent (67). Trapping of the blastocyst may occur if artificial gaps are too small. A link between gap size and monozygotic twinning is illustrated by recent findings after embryo biopsy. The rate of monozygotic twinning was not increased compared to the frequency in the natural population (68). Gaps produced for biopsy are a factor 2 or 3 times larger than those produced for assisted hatching. Similarly, no monozygotic twins have been found after transferring blastocysts from which the zona pellucida was removed (Coughlin Wagner and Maravilla, Highland Park, Chicago, personal communication).

Though our practice indicates that assisted hatching improves implantation, controversy continues to impede its widespread and dependable application with as many studies finding no effect as studies that do. In the first meta-analysis (69), 20 studies were considered, but only 14 were included in a meta-analysis as their information came from prospective

randomized controlled studies or retrospective studies with matched controls. Nevertheless, a significant overall benefit of assisted hatching was demonstrated, especially for older women. Other meta-analysis systems have not added to this understanding (52–56).

More than 10 years ago, we investigated the benefits of creating relatively large openings (15–20 μm on the inside to 30 to 50 μm on the outside) by zona drilling with acidified Tyrode's solution in the zonae of day 3 embryos undergoing initial compaction (57). It was shown that zona drilling increased the rate of implantation in patients whose embryos had thick zonae and others in whom embryos developed slowly. The procedure was most beneficial in patients with elevated basal follicle-stimulating hormone (FSH) levels or all those older than 38. This technique of selective assisted hatching has been implemented in patients. Implementation is dependent on individual embryonic variables, maternal age, previous history of the patient, and basal follicle-stimulating hormone (FSH) levels. Current policy extends assisted hatching to many patients, both IVF and ICSI, except those in whom traumatic transfers are anticipated and in others who simply do not consent to the procedure.

A number of programs have simplified selection criteria by using a patient age cut-off limit. Others apply assisted hatching to all patients who have failed IVF or ICSI before or whom are otherwise considered to have a lowered chance of becoming pregnant. The initial learning curve with this technique must be emphasized because individual and team results may improve considerably after experience.

Selection for Assisted Hatching

The guidelines for selecting individual embryos for assisted hatching are dependent on many variables. There are some hypothetical cut-off levels according to maternal age, elevated FSH, zona thickness, percentage fragmentation, and number of blastomeres, above or below which the chance of implantation is considerably reduced. Yet, all things being equal, the selection process remains a clinical decision and not a scientific one. The suggested guideline is that embryos from patients with elevated basal FSH levels are always manipulated regardless of other evaluations. With few exceptions, embryos from patients 38 years or older can also be zona-drilled. A truly thick zona is defined as having a mean zona pellucida (ZP) higher than 18 μm, but this number is dependent on the duration of follicular stimulation and maternal age as well as the instrument of observation. Such embryos can also be zona-drilled, regardless of other parameters.

Assisted hatching can also be performed on embryos which have 15% or more extra-cellular fragments or develop slowly. Failed patients are also considered for assisted hatching when their embryos were never zona-drilled before and had an apparently normal embryo transfer.

Techniques for Zona Opening: Acidified Solution

The use of acidified Tyrode's solution is widespread also because of its application for embryo biopsy. The diameter of the needle that deposits the acidified solution ranges from 10 to 12 μm. Although heated stages can be used, it is unknown what the optimal temperature should be while this type or any other embryo micromanipulation is performed. The micro-needle is front-loaded with acidified Tyrode's solution before each hatching event using precisely controlled suction. This type of control is especially important because the meniscus of the acidic fluid cannot be controlled easily.

The embryo should be pre-aligned only with the holding pipette, so that an open area between blastomeres, or an area of unusually large perivitelline space, or an area with a concentration of fragments is directly subjacent to the region to which the acidified solution shall be applied (usually the three o'clock position). In this way, the small amount of acidified solution that is expelled into the perivitelline space does not come into immediate contact with the surface of a blastomere before it is aspirated back into the hatching needle. Once the embryo is appropriately positioned with the use of the single tool, the micropipette filled with acidified solution is lowered into the medium and brought adjacent to the target area as fast as possible.

This is done in order to avoid dilution of acidified solution with medium of normal pH, as its release on the zona will jeopardize the embryo without affecting the zona pellucida. The key to successful assisted hatching is to minimize exposure to low pH. Most of the individual elements of the procedure are designed to produce a gap in the zona pellucida while minimizing the impact of the exposure to acidified solution on the embryo. As the acidified Tyrode's solution is aspirated through a needle of very small diameter, considerable residual suction (lower pressure) exists in the hatching needle even after it is removed from the reservoir drop of acidified Tyrode's. When the hatching needle enters the droplet of medium containing the embryo to be hatched, this residual suction will cause culture medium to be aspirated. A column of neutral pH medium will therefore be at the tip of the hatching needle, and the upstream acidified Tyrode's solution will likely be diluted.

In order to prevent residual suction while aspirating culture medium, the system should be prepared in advance so that the hatching needle will be in precisely the correct position relative to the embryo when it is lowered into the medium droplet. There should be no more than a two-second delay between the time the hatching needle enters the drop until the initiation of hatching. Also, a slight positive pressure in the hatching needle, applied as soon as the needle breaks the surface of the drop when it is lowered, will serve to counteract the residual suction. The embryologist should cease the procedure if the thinning aspect is not immediate.

The reduced pH solution should be expelled forcefully, so that the hole is made as quickly as possible and the time of exposure to the acidified solution is minimized. The total time necessary to breach the zona should not exceed a couple of seconds; most zonae will yield in fewer than five seconds. The needle should be applied directly to the zona pellucida, and the area to be opened should be massaged while the hole is being made, with the narrowest point being the inside of the zona. The massaging motion should allow the creation of a hole that is nearly rectangular. The inside layer of the zona pellucida is frequently more resistant to reduced pH than the outer layers. Care should be taken, therefore, to assure that zona breakthrough occurs over a sufficiently wide area of at least 20 μm, and not at a single small point.

As soon as the zona is breached, the flow through the assisted hatching needle must be immediately reversed. All of the expelled acidified solution should be aspirated, especially any and all solution that may have entered the perivitelline space. In any event, the embryo should be simultaneously moved to another area of the droplet, away from the area of reduced pH. The use of a mouth-controlled suction tube for assisted hatching may be preferable over other methods, to allow the instantaneous reversal of flow through the hatching needle as soon a break-through of the zona occurs.

Assisted Hatching with Laser

The non-contact infrared laser has emerged as the methodology perhaps best suited to mammalian zona-cutting applications (70). Several commercial systems are now available with Food and Drug Administration (FDA) permits using IR diode lasers, and these have been put to use in human clinical embryology procedures such as assisted hatching and biopsy. The appropriateness of some of the basic models investigating the infrared delivery system must be questioned, however, and particularly localized effects such as heat have only been assessed in other laser systems [for review see Malter et al. (71)]. Early and clinical studies have been generally hampered by lack of appropriate controls. FDA studies have been conducted, but the study designs have not always been optimal as results between laser-manipulated embryos and non-manipulated controls were compared (72). This confounds the effect of zona opening per se with any effects of the laser. Comparisons need to be made between the laser technique and other standard clinical methodology for opening the zona by mechanical or chemical means.

The problem with the more recent studies using laser applications is that only the efficacy has been questioned and safety has been evaluated in terms of pregnancy rates. The results reported appear promising, apparently demonstrating simple, repeatable, and appropriate zona ablation with no obvious detrimental effects, at least none that are reported (72,73). In some studies, implantation and clinical pregnancy rate may have been

increased following laser-mediated assisted hatching and a relatively large group of healthy babies have now been born. The positive results and efficacy reported for laser-based zona cutting have been intriguing; however, basic safety studies have been rare and those that exist (71) are rarely documented by researchers investigating clinical applications. Time will tell whether these tools are safe and whether improved pregnancy rates can be sustained.

Removal of Fragments and Lysed Cells After Cryopreservation

Once the gap in the zona pellucida is made, small fragments or lysed cells may be removed by aspiration with the hatching needle. The clinical benefit of this procedure is still debated (74,75), although the removal of lysed cells from thawed embryos is now clinically applied in a number of clinics with promising results (76,77). The technique requires masterly skill to remove all or most fragments and lysed material from an embryo using a single hole without causing damage. Great caution should be exercised because even the slightest touch of the hatching needle on the membrane of a blastomere can result in the loss of membrane integrity.

The use of a 12 μm diameter needle is preferred for this activity, compared to the approximate 10 μm of a regular assisted hatching needle. Suction should be instantly ceased if it appears that the membrane of a blastomere is reacting to the suction in any way. Some fragments and lysed debris may be firmly attached to blastomeres. In such cases, removal may be counter-productive. Removing fragments and lysed debris can be time-consuming and should be done gently and patiently. Continuous changes of focusing adjustment on target material and adjacent blastomeres are necessary because fragments are not all in the same plane as the pipette-tip and the artificial opening. The zona should be turned after removal of some fragments, as a different angle increases the likelihood that more fragments can be removed. Fragments in between blastomeres and those opposite the aperture should be removed last of all. Removal of fragments from areas between cells is especially advantageous because it improves cell–cell interaction during compaction (74).

Fragment and debris removal should only be practiced at the highest magnification and using a state-of-the-art interference microscope. Continuous refocusing and moving the zona around while determining the best approach are essential to this technique, including awareness of the distinct patterns of fragmentation. Most of the benefits of assisted hatching and fragment removal lie in the production of the artificial gap in the zona pellucida. The benefit derived from fragment removal over and above that of assisted hatching alone is real, but modest in comparison. When only a few fragments exist on the periphery of an embryo, it is probably unwise to spend much time attempting to remove them. When larger numbers of fragments are present, especially in an orientation that may interfere with

normal cell–cell contact and impede compaction, it makes sense to take more time to remove them.

The results of fragment removal are best weighed by assessing pregnancy rates of patients by grouping average rates of fragmentation of the replaced embryos prior to fragment removal. Even patients with the worst embryos must have at least a 30% chance of becoming pregnant. It is likely that this result is positively affected by the procedure, as well as by the expertise of the team.

Alikani and co-workers in 1993 were the first to explore the possibility of removing degenerate blastomeres from embryos before compaction using a mouse model (75). They also described that clinical pregnancies were established after removing lysed cells from human embryos but did not demonstrate efficacy. This was done recently by comparing datasets of embryos transferred with lysed cells remaining inside their zonae to embryos whose lysed cells were removed (77). A remarkable improvement in implantation was achieved.

Monozygotic Twinning and Assisted Hatching

The incidence of monozygtic twinning is quadrupled after follicular stimulation with or without assisted reproduction (68). Not all aspects of ART have been associated with monozygotic twinning. Follicular stimulation, poor embryo development, assisted hatching, and blastocyst development have all been described as risk factors, yet evidence of serious direct associations is largely missing. It is likely that, in the case of blastocyst transfer, the association is related to changes in the zona pellucida or the ability of the embryo to prepare for hatching, yet this is possibly related to subtle aspects of embryo culture and should be transient with improving conditions. Also, identical twinning may be reduced by removing the zona at this point mechanically or enzymatically or by performing a partial yet vigorous opening procedure. The association between assisted hatching and monozygotic twinning is complicated by confounding factors such as selection of patients and embryos. Again, the association between monozygotic twinning and micromanipulation may be because of technical variations; a more constraining zona opening is likely to lead to trapped or split embryos (65). This is illustrated by the observation that only 1/140 (0.7%) embryos that were biopsied had monozygotic twins (68). A larger zona opening is therefore likely to reduce nearly all factors associated with trapping and splitting, indicating that the association between assisted hatching and identical twinning is of a technical nature.

EMBRYO BIOPSY

PGD can now be performed at nearly any stage between egg maturation and cavitation of the embryo (1,78,79). The validity of PGD and the scope of

applications in fertile and infertile patients will be discussed elsewhere as this is a complex topic that requires broad discussion. The general interest in the biopsy technique itself and the understanding of aspects of the culture system that may play a role during micromanipulation for PGD is rather limited. Few studies have emphasized these aspects in spite of a genuine and broad interest in the genetic aspects of PGD. Here, biopsy of the cleaved embryo on day 3 will be discussed briefly, as this is still the method preferred by most clinics that are interested in PGD. There are basically three ways of opening the zona for embryo biopsy at the cleavage stage and two ways of extracting a blastomere through the artificially created gap (80–84). The zona can be opened mechanically, but this is still rarely applied, via the use of acidified solution (see assisted hatching section above) or using a laser. The targeted blastomere can either be extracted using a biopsy needle that absorbs the cell partially or completely and releases it elsewhere in the droplet or it can be released from the embryo using pressure on the zona pellucida using a tool that does not enter the zona pellucida. The latter is a single tool technique that is often used in combination with a laser. Surprisingly, there are no randomized studies that compare any of these techniques, although serial observations have been described (84). The circumstances of biopsy have not been studied in great detail. There is no information available from the literature on the optimal use of Ca- and Mg-free medium and any safety aspects in spite of its widespread use. There is consensus about its technical advantages because blastomere survival is clearly enhanced. There is no information available about the use of buffered culture systems and the optimal temperature. There is also no appropriate guideline for targeting a certain blastomere, although there is consensus that the blastomere should not be dividing and should have a single clear nucleus. It is unsure if there is already a predisposition of blastomeres to the inner cell mass and trophoblast at the eight-cell stage. From cryopreservation studies involving models of cell survival, it appears that such an early allocation is either missing or has little clinical effect after biopsy (85,86).

The most important question regarding embryo biopsy is the possible effect on further development and efficacy if an associated genetic diagnosis was not performed. In other words, does a single blastomere biopsy affect the embryo and is this loss compensated in one way or another by the subsequent genetic diagnosis? Certainly, the notion that PGD would be successful and result in acceptably high pregnancy rates after testing for single gene disorders was assumed many years ago, but clinical practice has been shown to be disappointing. The effects of cell loss after biopsy can be tested by performing prospective analysis of intact and biopsied embryos without genetic diagnosis, but such experiments may be considered ethically challenging. The proposition that blastocysts obtained after single-cell biopsy have cell numbers proportional to their initial cell count is often being

quoted as showing that the embryo can loose a cell; however, those experiments were done with well developing embryos from young donors (87). IVF practice shows that the number of cells on day 3 is not an average of eight, but a number that is lower and dependent on patient selection and other factors. In our EggCyte database with over 80,000 embryos, the average cell number on day 3 is not 8.0 but 6.7.

A possible estimate of the effect of cell number on viability can be derived from experience of cryopreservation of cleaved embryos and associated cell loss. It has been suggested that loss of viability after freezing is proportional to the number of cells lysed (85,86). For instance, if the expected implantation is 20%, then the loss of one cell from an 8-cell embryo would result in an implantation equal to $7/8 \times 20 = 17.5\%$. If the embryo has only 6 cells at freezing, a single cell loss would translate to $5/6 \times 20 = 16.7\%$. This means that if we postulate that there is no effect of assisted hatching in a given IVF program and if the result of biopsy is similar to that of cell lysis after thaw, any genetic diagnosis would have to make up for this loss in implantation potential. Obviously, this only applies to genetic diagnosis involving assessment of embryo normalcy. The loss in implantation potential could be very substantial if one considers the true average cell number and not automatically assume that all embryos have 8 cells on day 3. Again, if the expected outcome would be an implantation rate of 20%, the implantation rate after single cell biopsy of embryos from our laboratory (average cell number $= 6.7$) would be $5.7/6.7 \times 20 = 17.0\%$. The genetic diagnosis would have to make up for this loss in implantation potential. In our clinical experience of PGD for infertility involving the use of 5 to 9 chromosomes probes, we have demonstrated a significant increase in implantation when selecting patients because of advanced maternal age as well as repeated pregnancy loss (88,89). It must be noted that these procedures were performed using a single-cell biopsy. It has been advocated by others, particularly the Brussels team, that the number of cells extracted should be at least two and not one (90,91). This policy difference is based on the assumption that aneuploidy testing is another form of prenatal diagnosis and that the negative error rate should be as low as after clinical prenatal genetic diagnosis. Opinions differ in this respect because we consider aneuploidy testing as an additional way of selecting embryos for transfer. In the context of this policy, PGD becomes yet another useful tool for embryologists guiding them in their decision to choose the embryos with the highest likelihood of viability. Indeed, considering the concept of implantation potential and associated cell loss described here, biopsy of two cells would be double as detrimental as that of a single cell extraction. In this respect and using our model, implantation rates could drop as low as 14% (again presuming that the expected rate was 20%). Interestingly, in two recently published clinical trials by the Brussels team, implantation rates in the PGD for aneuploidy and control groups were similar, indicating that the genetic diagnosis

had made up for the initial damage to the biopsied embryos, yet the net result was disappointing (90,91). In conclusion, considering the effects of embryo biopsy and cell loss after freezing, it is recommended that clinical teams restrict the number of extracted cells to one and inform patients of potential errors. The latter figure is less than 5% after biopsy of a single cell in our work. Certainly, this number must be acceptable if one agrees with the notion that PGD is not prenatal genetic diagnosis.

MORAL, ETHICAL, AND POLITICAL CONSIDERATIONS

The debate on the ethics and morals of invasive manipulation of human gametes and embryos reached an unexpected apex in the spring of 1997 with the birth of a wonderfully smiling sheep called Dolly in Scotland, and once again with the creation in 2005 of human embryonic stem-cells (hESC) from somatic cells of diseased Korean patients by a process euphemistically called therapeutic nuclear transplantation (92,93). At the time of writing of this chapter, the latter group of researchers headed by Dr. Hwang is under investigation for fraud by Korean authorities and an investigation of Dr. Gerry Schatten's role in the affair is under scrutiny from the University of Pittsburgh. Korean sentiments have reached such an emotional response that American–Korean relations may have been affected, hopefully not perpetually. It is not the first time in history that a scientific finding has had major political repercussions. The consequences of Galileo's steadfast "and yet it moves" when referring to planet earth not being "fixed" was still felt in shaken-up Europe hundreds of years later. The political answers to biological phenomena and associated tools that can be applied in human assisted reproduction are being considered by nearly all governments, often before technologies become realistic, using emotional and religious considerations rather than common sense. The largely negative global response to the creation of children heteroplasmic in a non-coding region of the mitochondrial genome in 2001 testifies to the fear among most people regarding new medical technology. The fact that the 43rd president of the United States first public speech after eight months in office was about prohibiting the further creation of hESCs shows the enslavement of secular governments for contemporary Galileo-era-like limitations imposed by organized religion. The field of reproductive cell surgery apparently causes great concern among lawmakers and ethicists alike, especially as it involves the tools enabling scientists to perform nuclear transplantation. Laws were passed in haste in several countries banning cloning after Dolly's birth, but a few more contemplative lawmakers pointed at the potential benefits that cloning technology could foster and assumed that further evaluation was warranted. Yet to date, many countries prohibit the use of nuclear transplantation, sometimes even in animal models. Prime examples of advantages to be gained

are obvious in this chapter and throughout this book. Transferring a nucleus into an oocyte, such as is the case with ICSI, is cloning-derived technology. This is also apparent as reproductive specialists have attempted to transfer haploid sperm precursor cells. It is likely that, while attempting to select round haploid spermatids, the embryologists may select a somatic cell, with similar morphologic resemblance, which is usually present in the sperm preparation. It is actually possible that human embryos have already been accidentally created in this way. Moreover, the chance of spontaneous embryo splitting (a simple form of embryo duplication) is likely to be enhanced following certain forms of micromanipulation and assisted hatching. Removing cells from embryos during PGD (a technology now prohibited in several European countries) is yet another application where cloning technology has given us an advantage. Another example is cytoplasmic transfer, where in the most extreme form, nuclei can be exchanged between eggs or zygotes, in order to fully transplant the ooplasmic component. This is the most likely strategy that embryologists will follow when assisting women with mitochondrial disease to conceive, a notion that has been given the green light, at least in a pre-clinical investigative context by the human fertilization and embryology authority in the UK. The opportunity to treat infertility and prevent genetic disease in other generations should not be confused with or mistaken for cloning or alteration of the genome in an as yet undefined and possibly eugenic direction (genetic engineering). Nothing could be further from the truth. These procedures aim to normalize development by manipulation of compromised components other than the cells' nuclear genetic system; they aim only to provide or enhance the natural elements and conditions used by the nuclear genetic system for development when they are believed to be absent or somehow lacking in substance.

Patients should always be fully informed and provide written consent to all and any of the procedures described here. Investigative protocols such as these must be considered and approved by an appropriate scientific and ethical board. Successful duplication of the procedures depends in great measure on mastering the skills of micromanipulation and the experience and expertise of the individual embryologist, just as the success of advanced surgery depends upon the skill of the surgeon. This appears to be especially the case with procedures such as assisted hatching and fragment removal. We believe that the field of micromanipulation in embryology is just as exciting as that of surgery, perhaps more so, and that it may ultimately lead to widespread use of some of the techniques described here and, of course, it is to be expected that new and perhaps surprising applications will be discovered to overcome not only infertility, but also genetic disease. The timeline for this is not controlled by patients or their doctors. It is not dependent on scientists or their lack of funding. It is almost entirely constrained by the aptitude of powerful religious extremists.

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REFERENCES

1. Handyside AH, Kontogianni EH, Hardy K, Winston RML. Pregnancies from biopsied human preimplantation embryos sex by Y-specific DNA amplification. Nature 1990; 344:378.
2. Cohen J, Elsner C, Kort H, et al. Impairment of the hatching process following IVF in the human and improvement of implantation by assisted hatching using micromanipulation. Hum Reprod 1990; 5:7.
3. Palermo G, Joris H, Devroey P, Van Steirteghem A. Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte. Lancet 1992; 340:17.
4. Cohen J, Garrisi GJ, Congedo-Ferrara TA, Kieck K, Schimmel, TW, Scott R. Cryopreservation of single human sperm cells. Hum Reprod 1997; 12:994.
5. Cohen J, Scott RT, Schimmel T, Levron J, Willadsen SM. Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. Lancet 1997; 350:186.
6. Levron J, Willadsen SM, Shimmel T, Cohen J. Cryopreservation of activated mouse oocytes and zygote reconstitution after thaw. Hum Reprod 1998; 13(suppl 4):61–70.
7. Cummins JM. Can and should human embryos be "rescued" from developmental demise? In: Van Blerkom J, Gregory L, eds. Methods and Biological Basis. Norwell, MA: Kluwer Academic Publishers, 2004:555–575.
8. Craft I, Bennett V, Nicholson N. Fertilising ability of testicular spermatozoa. Lancet 1993; 342:864.
9. Schoysman R, Vanderzwalmen P, Segal-Bertin G, van de Casseye M. Successful fertilization by testicular spermatozoa in an in vitro fertilization programme. Hum Reprod 1993; 8:1339.
10. Hopps CV, Goldstein M, Schlegel PN. The diagnosis and treatment of the azoospermic patient in the age of intracytoplasmic sperm injection. Urol Clin North Am 2002; 29:895.
11. Schlegel P. Physiologic consequences of TESE. Hum Reprod 1996; 11:159.
12. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P. High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa from testicle biopsy. Hum Reprod 1995; 10:148.
13. Fisher R, Baukloh V, Naether OGJ, Sshulze W, Salzbrunn A, Benson DM. Pregnancy after intra-cytoplasmic sperm injection of spermatozoa extracted from frozen-thawed testicular biopsy. Hum Reprod 1996; 11:2197.
14. Desai NN, Blackmon H, Goldfarb J. Single sperm cryopreservation on cryoloops: an alternative to hamster zona for freezing individual spermatozoa. Reprod Biomed Online 2004; 9:47.

15. Hsieh Y, Tsai H, Chang C, Lo H. Cryopreservation of human spermatozoa within human or mouse empty zona pellucidae. Fertil Steril 2000; 73:694.
16. Levi-Setti PE, Albani E, Negri L, Cesana A, Novara P, Bianchi S. Cryopreservation of a small number of spermatozoa in yolk-filled human zonae pellucidae. Arch Ital Urol Androl 2003; 75:195.
17. Montag M, Rink K, Dieckmann U, Delacretaz G, van der Ven H. Laser-assisted cryopreservation of single human spermatozoa in cell-free zona pellucida. Andrologia 1999; 31:49.
18. Pieczenik G, Garrisi GJ, Cohen J. Inhibition of spermatozoa-zona pellucida binding by a combinatorially derived peptide from a synthetic target. Reprod Biomed Online. In press.
19. Bongso A, Jarina AK, Ho J, Ng SC, Ratnam SS. Comparative evaluation of three sperm-washing methods to improve sperm concentration and motility in frozen-thawed oligozoospermic and normozoospermic samples. Arch Androl 1993; 31:223.
20. Verheyen G, Pletincx I, Van Steirteghem A. Effect of freezing method, thawing temperature and post-thaw dilution/washing on motility (CASA) and morphology characteristics of high quality human sperm. Hum Reprod 1993; 8:1678.
21. Sawetawan C, Bruns ES, Gail SP. Improvement of post-thaw motility in poor quality human semen. Fertil Steril 1993; 60:706.
22. Walmsley R, Cohen J, Ferrara-Congedo T, Reing A, Garrisi J. The first births and ongoing pregnancies associated with sperm cryopreservation within evacuated egg zonae. Hum Reprod 1998; 13(suppl 4):61–70.
23. Barritt J, Willadsen S, Brenner C, Cohen J. Cytoplasmic transfer in assisted reproduction. Hum Reprod Update 2001; 7:428.
24. Tzeng C, Hsieh S, Chang N. Pregnancy derived from mitochondrial transfer (MIT) into oocyte from patient's own cumulus cells (cGCs). Fertil Steril 2001; 76(suppl 3S):O-180.
25. Tzeng CR, Hsieh RH, Au HK, Yen YH, Chang SJ, Cheng YF. Mitochondria transfer (MIT) into oocyte from autologous cumulus granulosa cells (cGCs). Fertil Steril 2004; 82(suppl 2):S53.
26. Kong LH, Liu Z, Li H, et al. Mitochondria transfer from self-granular cells to improve embryos' quality. Zhonghua Fu Chan Ke Za Zhi 2004; 39:105.
27. Lanzendorf SE, Mayer JF, Toner J, Oehninger S, Saffan DS, Muasher S. Pregnancy following transfer of ooplasm from cryopreserved-thawed donor oocytes into recipient oocytes. Fertil Steril 1999; 71:575.
28. Huang C, Cheng T, Chang H, et al. Birth after the injection of sperm and the cytoplasm of tripronucleate zygotes into metaphase II oocytes in patients with repeated implantation failure after assisted fertilization procedures. Fertil Steril 1999; 72:702.
29. Malter HE, Cohen J. Ooplasmic transfer: animal models assist human studies. Reprod Biomed Online 2002; 5:26.
30. Barritt J, Brenner CA, Malter HE, Cohen J. Rebuttal: inter-ooplasmic transfers in humans. Reprod Biomed Online 2001; 3:42.
31. Schultz RM, Williams CJ. The science of ART. Science 2002; 296:2188.
32. St John JC. Ooplasm donation in humans: the need to investigate the transmission of mitochondrial DNA following cytoplasmic transfer. Hum Reprod 2002; 17:1954.

33. Templeton A. Ooplasmic transfer—proceed with care. N Eng J Med 2002; 346:774.
34. Barritt JA, Brenner CA, Malter HE, Cohen J. Mitochondria in human offspring derived from ooplasmic transplantation. Hum Reprod 2001; 16:513.
35. Hawes SM, Sapienza C, Latham KE. Ooplasmic donation in humans: the potential for epigenic modifications. Hum Reprod 2002; 17:850.
36. Opsahl MS, Thorsell LP, Geltinger ME, Iwaszko, MA, Blauer KL, Sherins RJ. Donor oocyte cytoplasmic transfer did not enhance implantation of embryos of women with poor ovarian reserve. J Assist Reprod Genet 2002; 19:113.
37. Warburton D. Biological aging and the etiology of aneuploidy. Cytogenet Genome Res 2005; 111:266.
38. Cohen J, Scott R, Alikani M, et al. Ooplasmic transfer in mature human oocytes. Mol Hum Reprod 1998; 4:269.
39. Takeda K, Tasai M, Iwamoto M, et al. Microinjection of cytoplasm or mitochondria derived from somatic cells affects parthenogenetic development of murine oocytes. Biol Reprod 2005; 72:1397.
40. Alikani M, Schimmel T, Willadsen SM. Cytoplasmic fragmentation in activated eggs occurs in the cytokinetic phase of the cell cycle, in lieu of normal cytokinesis, and in response to cytoskeletal disorder. Mol Hum Reprod 2005; 11:335.
41. Tully LA, Parsons TJ, Steighner RJ, Holland MM, Marino MA, Prenger VL. A sensitive denaturing gradient-gel electrophoresis assay reveals a high frequency of heteroplasmy in hypervariable region 1 of the human mtDNA control region. Am J Hum Genet 2000; 67:432.
42. Petri B, von Haeseler A, Paabo S. Extreme sequence heteroplasmy in bat mitochondrial DNA. Biol Chem 1996; 377:661.
43. Casane D, Dennebouy N, de Rochambeau H, Mounolou JC, Monnerot M. Non-neutral evolution of tandem repeats in the mitochondrial DNA control region of lagomorphs. Mol Biol Evol 1997; 14:779.
44. Kenyon L, Moraes C. Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids. Proc Nat Acad Sci USA 1997; 94:9131.
45. Latham K, Sapienza C. Localization of genes encoding egg modifiers of paternal genome function to mouse chromosomes one and two. Development 1998; 125:889.
46. Latham K, Kutyna K, Wang Q. Genetic variation in trophectoderm function in parthenogenetic mouse embyos. Dev Genet 1999; 24.
47. Killian J, Nolan C, Wylie A. Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. Hum Molec Genet 2001; 10:1721.
48. Schieve LA, Rasmussen SA, Buck GM, Schendel DE, Reynolds MA, Wright VC. Are children born after assisted reproductive technology at increased risk for adverse health outcomes? Obstet Gynecol 2004; 103:1154.
49. Gosden R, Trasler J, Lucifero D, Faddy M. Rare congenital disorders, imprinted genes, and assisted reproductive technology. Lancet 2003; 361(9373):1975.
50. Bonduelle M, Liebaers I, Deketelaere V. Neonatal data on a cohort of 2889 infants born after ICSI and of 2995 infants born after IVF (1983–1999). Hum Reprod 2002; 17:671.
51. Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R, Horsthemke B. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. J Med Genet 2005; 42:289.

52. Edi-Osagie E, Hooper L, Seif MW. The impact of assisted hatching on live birth rates and outcomes of assisted conception: a systematic review. Hum Reprod 2003; 18:1828.
53. Sallam HN, Sadek SS, Agameya AF. Assisted hatching—a meta-analysis of randomized controlled trials. J Assist Reprod Genet 2003; 20:332.
54. Edi-Osagie EC, Hooper L, McGinlay P, Seif MW. Effect(s) of assisted hatching on assisted conception (IVF & ICSI). Cochrane Database Syst Rev 2003; CD001894.
55. Sallam HN. Assisted hatching. Minerva Ginecol 2004; 56:223–234.
56. Seif M, Edi-Osagie E, Farquhar C, et al. Assisted hatching on assisted conception (IVF & ICSI). Cochrane Database Syst Rev 2005; 19:CD001894.
57. Cohen J, Alikani M, Trowbridge J, Rosenwaks Z. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. Hum Reprod 1993; 8:599.
58. Wright G, Jones A. In: Van Blerkom J, Gregory L, eds. Assisted Hatching in Clinical IVF. Norwell, MA: Kluwer Academic Publishers, 2004:441.
59. Practice Committee of the American Society for Reproductive Medicine. The role of assisted hatching in in vitro fertilization: a review of the literature. Fertil Steril 2004; 82(suppl 1):S164.
60. Magli MC, Gianaroli L, Ferraretti AP. Chromosomal abnormalities in embryos. Mol Cell Endocrinol 2001; 183(suppl 1):S29.
61. Liu H-C, Cohen J, Alikani M, Noyes N, Rosenwaks, Z. Assisted hatching facilitates earlier implantation. Fertil Steril 1993; 60:871.
62. Petersen CG, Mauri AL, Baruffi RL, et al. Implantation failures: success of assisted hatching with quarter-laser zona thinning. Reprod Biomed Online 2005; 10:224.
63. Cohen J, Malter H, Elsner C, Kort H, Massey J, Mayer MP. Immunosuppression supports implantation of zona pellucida dissected human embryos. Fertil Steril 1990; 53:662.
64. Nakayama T, Fujiwara H, Yamada S, Tastumi K, Honda T, Fuji S. Clinical application of a new assisted hatching method using a piezo-micromanipulator for morphologically low-quality embryos in poor-prognosis infertile patients. Fertil Steril 1999; 71:1014.
65. Cohen J, Feldberg D. Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. Molec Reprod Dev 1991; 30:70.
66. Cieslak J, Ivakhnenko V, Wolf G, Sheleg S, Verlinsky Y. Three-dimensional partial zona dissection for preimplantation genetic diagnosis and assisted hatching. Fertil Steril 1999; 71:308.
67. Lyu QF, Wu LQ, Li YP, et al. An improved mechanical technique for assisted hatching. Hum Reprod 2005; 20:1619.
68. Alikani M, Cekleniak NA, Walters E, Cohen J. Monozygotic twinning following assisted conception: an analysis of 81 consecutive cases. Hum Reprod 2003; 18:1937.
69. Brinsden P, Mascarenhas L, Avery S. Clinical benefits of assisted hatching. In: Gomel V, Leung PCK, eds. In Vitro Fertilization and Assisted Reproduction. Bologna, Italy: Monduzzi Editore, 1997:621.

70. Germond M, Nocera D, Senn A, Rink K, Delacretaz, G, Fakan S. Microdissection of mouse and human zona pellucida using a 1.48-microns diode laser beam: efficacy and safety of the procedure. Fertil Steril 1995; 64:604.
71. Malter HE, Schimmel T, Cohen J. Zona dissection by infrared laser: developmental consequences in the mouse, technical considerations, and controlled clinical trial. Reprod Biomed Online 2003; 3:117.
72. Primi MP, Senn A, Montag M, et al. European multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immunosuppressive/antibiotic treatment in different patient populations. Hum Reprod 2004; 19:2325.
73. Ebner T, Moser M, Tews G. Possible applications of a non-contact 1.48 microm wavelength diode laser in assisted reproduction technologies. Hum Reprod Update 2005; 11:425.
74. Alikani M, Sadowy S, Cohen J. Human embryo morphology and developmental capacity. In: Van Soom A, Boerjan, M, eds. Assessment of Mammalian Embryo Quality, Invasive and Non-invasive Techniques. The Netherlands: Kluwer Academic Publishers, 2002:1–24.
75. Alikani M, Olivennes F, Cohen J. Microsurgical correction of partially degenerate mouse embryos promotes hatching and restores their viability. Hum Reprod 1993; 8:1723.
76. Rienzi L, Nagy ZP, Ubaldi F, et al. Laser-assisted removal of necrotic blastomeres from cryopreserved embryos that were partially damaged. Fertil Steril 2002; 77:1196.
77. Nagy ZP, Taylor T, Elliott T, Massey JB, Kort HI, Shapiro DB. Removal of lysed blastomeres from frozen-thawed embryos improves implantation and pregnancy rates in frozen embryo transfer cycles. Fertil Steril 2005; 84:1606.
78. Verlinsky Y, Ginsberg N, Lifchez A, Valle J, Moise J, Strom CM. Analysis of the first polar body: preconception genetic diagnosis. Hum Reprod 1990; 5:826.
79. McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. Fertil Steril 2005; 84:1628.
80. Wilton LJ, Trounson AO. Biopsy of preimplantation mouse embryos: development of micromanipulated embryos and proliferation of single blastomeres in vitro. Biol Reprod 1989; 40:145.
81. Grifo JA, Boyle A, Fischer E, et al. Pre-embryo biopsy and analysis of blastomeres by in situ hybridization. Am J Obstet Gynecol 1990; 163:2013.
82. Grifo JA, Tang YX, Cohen J, Gilbert F, Sanyal MK, Rosenwaks Z. Pregnancy after embryo biopsy and coamplification of DNA from X and Y chromosomes. JAMA 1992; 12(268):727.
83. Inzunza J, Iwarsson E, Fridstrom M, et al. Application of single-needle blastomere biopsy in human preimplantation genetic diagnosis. Prenat Diagn 1998; 18:1381.
84. Joris H, De Vos A, Janssens R, Devroey P, Liebaers I, Van Steirteghem A. Comparison of the results of human embryo biopsy and outcome of PGD after zona drilling using acid Tyrode medium or a laser. Hum Reprod 2003; 18:1896.
85. Edgar DH, Bourne H, Jericho H, McBain JC. The developmental potential of cryopreserved human embryos. Mol Cell Endocrinol 2000; 27:69–169.

86. Edgar DH, Bourne H, Speirs AL, McBain JC. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. Hum Reprod 2000; 15:175.
87. Hardy K, Martin KL, Leese HJ, Winston RM, Handyside AH. Human preimplantation development in vitro is not adversely affected by biopsy at the 8-cell stage. Hum Reprod 1990; 5:708.
88. Munne S, Sandalinas M, Escudero T, et al. Improved implantation after preimplantation genetic diagnosis of aneuploidy. Reprod Biomed Online 2003; 7:91.
89. Munne S, Chen S, Fischer J, et al. Preimplantation genetic diagnosis reduces pregnancy loss in women aged 35 years and older with a history of recurrent miscarriages. Fertil Steril 2005; 84:331.
90. Staessen C, Platteau P, Van Assche E, et al. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. Hum Reprod 2004; 19:2849.
91. Platteau P, Staessen C, Michiels A, Van Steirteghem A, Liebaers I, Devroey P. Preimplantation genetic diagnosis for aneuploidy screening in patients with unexplained recurrent miscarriages. Fertil Steril 2005; 83:393.
92. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810.
93. Hwang WS, Roh SI, Lee BC, et al. Patient-specific embryonic stem cells derived from human SCNT blastocysts. Science 2005; 308(5729):1777.

15

Preimplantation Genetic Diagnosis

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INTRODUCTION

In 1990, preimplantation genetic diagnosis (PGD) was introduced as an experimental procedure to genetically screen human embryos during an in vitro fertilization (IVF) cycle (1,2). More than a decade later, PGD has become an established clinical procedure in assisted reproductive technologies with over 6500 PGD cycles performed worldwide, resulting in the birth of well over 1000 healthy babies and a pregnancy rate per transfer of approximately 24% (3). The safety of PGD is reflected in these comparable pregnancy rates with conventional IVF, as well as the equivalent incidence of birth abnormalities in the general population (4). PGD was initially performed for preexisting Mendelian-inherited monogenic disorders including X-linked disorders involving sex selection (1), cystic fibrosis (5), and Tay-Sachs disease (6). With the development of interphase single-cell fluorescent in situ hybridization (FISH) in the early 1990s, PGD has expanded to offer screening for chromosomal disorders including aneuploidy detection for clinically significant chromosomes (7,8) and translocations (9,10). PGD involves the molecular analysis of genetic material derived from oocytes or embryos during an IVF cycle. Only embryos identified as free of the indicated genetic disorder or chromosomal error are selected for transfer to the woman's uterus. Consequently, an established pregnancy is expected to be unaffected with respect to the indicated genetic testing.

SOURCE OF GENETIC MATERIAL

There are three different sources of genetic material potentially available for PGD: polar bodies from the initial conception, blastomeres from early cleaving embryos, and trophectoderm cells from the later stage blastocyst. A biopsy is performed to remove these cells for subsequent genetic analysis (11). Several procedures have been developed to create a hole in the zona pellucida including mechanically by conventional partial zona dissection (12), chemically using acid Tyrodes solution (13), or with the use of non-contact laser technology (14,15). The biopsy of these cells is predominantly performed under an inverted microscope with contrast optics using glass holding, needle, or suction micropipettes, and a set of micromanipulators attached to a pneumatic- or hydraulic-based system (11). The biopsy of polar bodies extruded by the oocyte or blastomeres from cleavage-stage embryos is considered safe on the basis of implantation and pregnancy rates reported in the literature that are comparable with conventional IVF (16).

Polar body biopsy can be performed preconceptionally to remove the first polar body or post-fertilization to remove either only the second polar body or both polar bodies simultaneously (Fig. 1) (17,18). Polar bodies are naturally extruded from the oocyte with no further role in the development of the future embryo. They each have a set of chromosomes that are complementary to those present in the oocyte. The first polar body is formed during meiosis (M) I of oogenesis and has a set of bivalent chromosomes, whereas the second polar body is formed during MII, after fertilization, and contains a haploid set of chromosomes. Both polar bodies give complementary diagnostic readouts and by deduction infer the genetic status of the oocyte. The main advantages of polar body biopsy include the

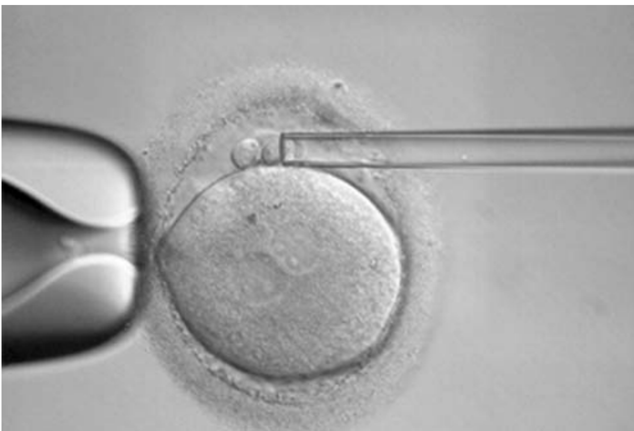

Figure 1 Polar body biopsy.

extra-embryonic nature of the polar bodies and the additional amount of time available for genetic analysis prior to embryo transfer. However, the major disadvantage of this technique is that the sex and paternal genotype are not available for the analysis, thus precluding polar body biopsy for the analysis of paternal mutations, gender determination, and chromosomal abnormalities arising from paternal meiosis. In addition, polar bodies undergo fragmentation rendering them often difficult to biopsy which can potentially lead to misdiagnosis if the embryologist is unable to retrieve all the polar body fragments.

Currently, the preferred stage for obtaining cells for genetic diagnosis is a blastomere biopsy of the cleavage-stage embryo performed at the 6–10-cell stage on day three post-fertilization (Fig. 2) (16). This allows for the retrieval of a blastomere containing both the maternal and paternal genomes. As these blastomeres are totipotent, the biopsy of 1–2 cells from the developing embryo does not seem to drastically reduce either the mass or contribution to the fetus, thereby not affecting the future viability of the embryo (19,20). Reports of ongoing comparable clinical pregnancy and implantation rates with conventional IVF confirm these earlier studies (4). The blastomere biopsy procedure can be performed by either gentle aspiration (Fig. 2) or by the nudge of the flow of biopsy media. Even if the embryo has begun compaction with gap junctions forming between the blastomeres, a blastomere biopsy can be performed after a short pre-incubation in calcium–magnesium-free media to reduce the cellular apposition. The major disadvantage of blastomere biopsy is the invasive nature of the procedure on the embryo itself along with the reduction in cell

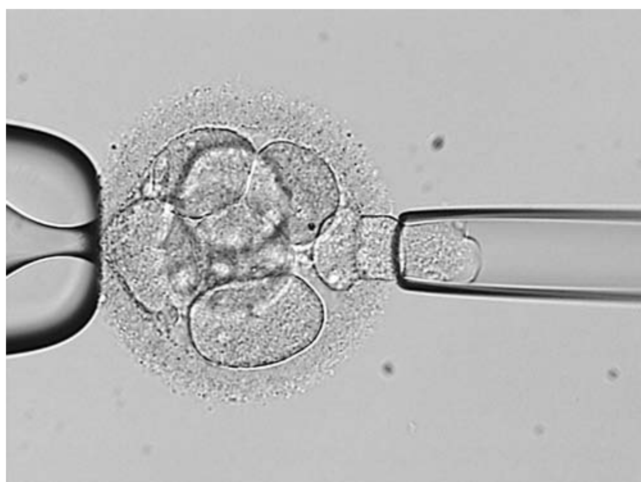

Figure 2 Blastomere biopsy.

number and the potential influence on further fetal development. There has been debate in the PGD field as to whether one or two cells should be biopsied. Clearly, the removal of two cells further reduces the cellular mass of the cleavage-stage embryo and may result in a reduction in developmental potential (21). Only a handful of studies have compared the outcome of one versus two biopsied cells, concluding that there were no decreases in implantation rates (22) with potentially fewer misdiagnoses (23). Ongoing prospective studies are underway to further address this question.

A human blastocyst, depending on the exact stage of development, can contain over 100 cells. Hence, the biopsy of 6–10 cells from the outer layer of trophectoderm is unlikely to have a detrimental effect on the blastocyst's mass or on the developing fetus that originates from the inner cell mass (ICM) (24–26). Prior to biopsy, the position of the ICM is identified so that the hole in the zona can be created on the opposite side of the blastocyst, reducing possible developmental and ethical concerns (Fig. 3). The cells are removed either mechanically by mild teasing using needles (24,27) or after herniation of the trophectoderm allowing biopsy by laser (Fig. 3) (28,29). The major advantage of this procedure is the larger amount of material available for the genetic testing, thereby increasing the reliability and accuracy of the diagnosis. However, the time for the analysis is limited to no more than 24 hours, as the blastocyst needs to implant at this stage. There are also uncertainties surrounding the genetic make up of the trophectoderm in relation to that of the ICM, the future-developing fetus. Due to these issues and the fact that some clinics prefer not to culture to the

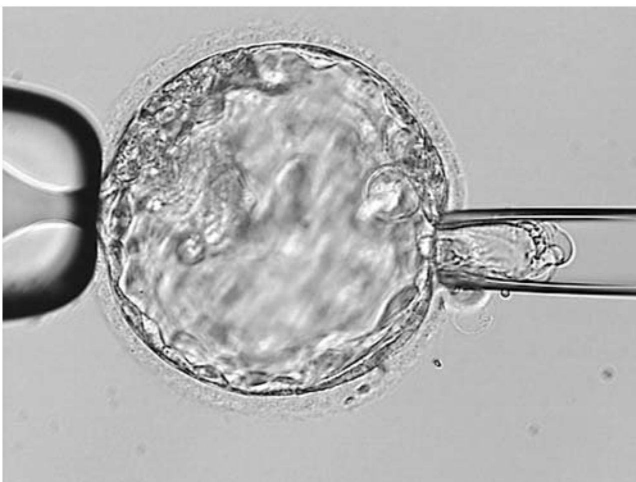

Figure 3 Trophectoderm biopsy.

blastocyst stage, only a limited number of IVF clinics perform blastocyst biopsy routinely. However, these clinics have reported ongoing clinical pregnancies and healthy babies (28,29).

PGD FOR MONOGENIC DISORDERS

The current experience of PGD for monogenic disorders exceeds more than 1500 cycles comprising over 50 different conditions and the birth of more than 300 unaffected children (3). PGD has been performed for autosomal-recessive (e.g., cystic fibrosis), autosomal-dominant (e.g., Huntington disease), and X-linked (e.g., Fragile X) inherited disorders (3). PGD has been established as an acceptable form of early prenatal diagnosis with the spectrum of conditions expanding with patient demand. The main motives behind couples seeking PGD are objection to potential therapeutic abortion (~47%), genetic risk coupled with low fertility (~32%), and repeated therapeutic abortions of identified affected fetuses (~26%) (30). In addition to conventional monogenic disorders, PGD is now requested for conditions such as late-onset predisposition disorders, blood group incompatibility, and human leukocyte antigen (HLA) matching (3,31).

In routine genetic diagnostic procedures, a starting template of at least 10 ng of DNA is usually available. However, a single cell contains only 6 pg of DNA (32) and has only two copies of each target locus. Some of the requirements that need to be addressed in PGD for monogenic disorders are the difficulties in the amplification of single-cell templates and the establishment of a procedure for high amplification efficiency and accuracy. A considerable amount of time and resources are required for the development of reliable and accurate single-cell diagnostic tests including the preliminary mutation workup. Careful experimental practices and suitable facilities including allocated equipment and vigilant quality control are essential (16). Specific and individual mutation-detection systems have been developed to capture and visualize the different DNA variants involved in monogenic disorders, including single base pair substitutions, deletions, insertions, duplications, and trinucleotide repeat expansions (3,4).

Polymerase chain reaction (PCR) is a common technique performed in PGD for monogenic disorders. It is a rapid, highly sensitive, and specific molecular technique that is capable of amplifying single copies of DNA template into large numbers with high fidelity (33). In a PCR reaction, several DNA sequences or loci can be independently amplified at the same time using multiple primer pairs in a technique called multiplex PCR. Once the DNA from the single cell has been amplified, there are numerous detection methods available to visualize the PCR products for the presence or absence of the specific DNA mutation or variant. The choice of the technique is usually dependent on the nature of the specific DNA mutation or variant and includes restriction endonuclease digestion (34,35), single-strand

conformational polymorphism (36), denaturant gradient gel electrophoresis (37), heteroduplex analysis (38), single nucleotide primer extension (39), and analysis of DNA fragment size (40,41). Fluorescent technology has further increased sensitivity of these detection methods resulting in the requirement of fewer PCR amplification cycles and greater reliability.

Real-time PCR is a more recent molecular technique that allows the mutation amplification and subsequent fluorescent detection procedure to be carried out in the same tube. Fluorescently tagged probes directed to either the normal or mutant sequence allow detection of the rate of amplification product accumulation to be measured directly by associated computer software as the PCR reaction proceeds (42,43). Unfortunately, there has been a slow uptake of this technology in PGD labs due to the enormous costs involved in purchasing the specialized equipment and consumables.

Several misdiagnoses have been reported by clinics around the world due to the complexity and sensitivity of single-cell PCR analysis (16). It is of vital importance that PGD relies on a positive result from the biopsied cell, thereby reducing the possibility of the transfer of affected embryos. The three main sources of potential misdiagnosis in PGD include external DNA contamination, complete amplification failure, and allele drop out (ADO).

External DNA contamination is a major problem due to the limited starting template and the large number of PCR amplification cycles required. Even at normal detectable levels, external DNA contamination may disguise or overwhelm a single cell and cause a misdiagnosis. The main laboratory contaminants include previously amplified PCR products accumulated in the laboratory and skin cells from the technician. The implementation of strict experimental practices and appropriate facilities will essentially minimize this risk including the following examples: a dedicated laminar flow hood with ultraviolet light to destroy any DNA by thymidine cross-linking, the isolation of all equipment for PGD use only, filtration and autoclaving of reagents, aliquots for storage, long sleeve lab gowns, caps and masks, and frequent glove changes (44). Pre-testing of all reagents and solutions prior to a clinical PGD case is essential to confirm the reliability of the test as well as for contamination prevention. Other potential sources of contaminants include the cumulus cells that surround the oocyte and excess sperm bound to the zona pellucida at the time of fertilization. These cells could accidentally be removed along with the polar body or blastomere during the biopsy procedure. It is therefore recommended for PGD of monogenic disorders that all oocytes be completely striped of their cumulus complex and that intracytoplasmic sperm injection is chosen as the method for fertilization.

It is also possible to detect contamination by the simultaneous PCR amplification of highly polymorphic DNA markers (45). This is similar to DNA fingerprinting techniques whereby highly polymorphic DNA markers allow the distinction between maternal and paternal alleles (46). As polymorphic DNA markers obey the laws of Mendelian inheritance, the

embryos of any couple can only inherit a specific combination of alleles at any particular locus (47). Therefore, an incorrect combination of alleles or the presence of extra foreign alleles is an indication that contamination has occurred. It has become increasingly common for PCR-based single-cell tests to include the amplification of several polymorphic DNA markers alongside the mutation loci used for diagnosis. If intragenic markers are chosen, they are linked in 100% disequilibrium to the gene of interest and are unlikely to be separated by recombination during meiosis. Hence, these polymorphic DNA markers have a dual purpose to recognize potential extraneous DNA contamination and act as a positive control for amplification (34,38).

Complete amplification failure of a mutation locus is observed in 5–10% of single cells. The source of the failure could be due to a number of factors, including failure to transfer the single cell into the PCR tube, an enucleate cell, degradation or loss of the target DNA sequence, and/or inefficient cell lysis (48,49). In contrast, ADO is defined as the amplification failure of either the maternal or paternal allele, giving the impression that a locus is homozygous. ADO has been observed to affect both parental alleles randomly. PGD involving dominant monogenic disorders is particularly vulnerable to misdiagnosis from ADO by the transfer of affected embryos that are incorrectly diagnosed as unaffected homozygous. One method to prevent misdiagnosis by ADO is the simultaneous amplification of intragenic polymorphic DNA markers in combination with the gene of interest. These markers would be inherited alongside the mutation locus providing additional loci for the detection of affected embryos. It is improbable that all loci amplified during a multiplex PCR reaction would be affected by ADO (38,50). The factors that cause ADO are yet to be completely elucidated; however, differences in PCR thermal cycling conditions, fragment size, incomplete cell lysis, the degradation of target template sequence, freezing and thawing, and poor specificity of primer pairs could possibly explain the variability in observed ADO rates (51).

The development of new reliable single-cell strategies, often for only one specific monogenic disorder, requires a major investment in resources, staff, finances, and time. Obviously, more adaptable and universal techniques are required in PGD that will allow a wider range of mutations to be concurrently investigated. One platform that may be able to achieve this goal is microarray technology. Specific sequences of DNA incorporating different mutations would act as probes on a microarray slide or chip, allowing hybridization between these known DNA probes and test DNA amplified from the single biopsied cell. Initial development of a specific cystic fibrosis deltaF508 array highlighted the diagnostic capability of microarrays for PGD (52). However, for this technology to be offered clinically, several issues need to be addressed including the reliable amplification of the whole genome from a single biopsied cell, a reduction in the complexity and time

for data analysis, and more cost-effective microarray platforms comprising DNA probes for numerous common monogenic disorders.

Whole genome amplification (WGA) is a technique aimed at maximizing the amount of information that can be obtained from a single cell or limited template. WGA theoretically involves the non-specific amplification of the entire genome, thereby increasing the amount of template for subsequent PCR reactions and multiple genetic analyses (53,54). There are several types of WGA protocols that have been developed to amplify DNA from small numbers of cells including primer extension preamplification (55), degenerate oligonucleotide primer PCR (56), and multiple displacement amplification (MDA) (57). Recently, MDA has been incorporated in clinical PGD for cystic fibrosis and β-thalassaemia resulting in two pregnancies (58). Some of the drawbacks to WGA methods include higher incidences of ADO, inaccurate size fragments, and inconsistent amplification of the whole genome (59,60). It is paramount that any WGA protocol incorporated in clinical PGD be reliable, accurate, and complete in the amplification of the entire human genome from a single cell.

PGD is considered an early form of prenatal diagnosis allowing high-risk couples to establish pregnancies free of the indicated genetic disorder. This technology is viewed as a positive contribution to the field giving couples early reassurance and avoidance of therapeutic abortion. Indications for PGD will continue to grow with patient demand and advancing technology. The use of PGD for non-medical indications, including HLA matching for siblings suffering lethal diseases such as leukemia and late-onset diseases such as cancer predisposition, are also likely to become more common. These non-medical indications have attracted media attention and passionate public debate concerning the ethics of "designer babies." In contrast, it has been argued that PGD for non-medical reasons highlights the love and commitment of couples to treat and prevent disease in their children and therefore should be viewed as an acceptable treatment.

CHROMOSOMAL ANEUPLOIDY SCREENING

Chromosomal analysis of human IVF embryos using single-cell interphase fluorescent in situ hybridization (FISH) was first developed to screen for embryo sex, allowing for the detection of the two sex chromosomes (7,61). Over time, the number of chromosomes for detection has increased significantly, allowing for screening of chromosomal aneuploidy in up to 9–10 chromosomes (62,63). Studies have shown that fetal chromosomal abnormalities are associated with human implantation failure and pregnancy loss (64,65). Therefore, chromosomal aneuploidy screening in PGD was introduced for IVF patients who are considered to be at increased risk of producing embryos with chromosomal abnormalities. These at-risk groups include advanced maternal age (>36 year), repeated miscarriages (RM), poor

IVF prognosis (>3 failed cycles), and couples who carry a chromosome rearrangement (such as translocations and inversions). Chromosomal aneuploidy screening of these patients' embryos should identify euploid embryos for the indicated chromosomes leading to a greater chance of implantation and clinical pregnancy. PGD for aneuploidy screening now accounts for the majority of PGD cycles worldwide, estimated at close to 5000 cycles (3). The benefit of PGD for aneuploidy screening has been reported by several groups showing an increase in implantation rates and decrease in miscarriage rates (31,62,63,66,67).

Single-cell interphase FISH is a rapid, reliable, and efficient technique capable of detecting up to 9–10 chromosomes in two rounds of hybridization on a single nucleus (62,63). Currently, fluorescent DNA probes for chromosomes X, Y, 13, 14, 15, 16, 18, 21, and 22 are being used in PGD for aneuploidy screening as they are involved in more than 50% of all chromosomal abnormal miscarriages (65). In the cases of chromosomal translocations, probes distal to the sites of chromosome breakage are used in addition to centromeric and proximal probes (68). The technical difficulties encountered in regards to selecting appropriate FISH probes and optimizing protocols for each couple's specific chromosome rearrangement are considerably time-consuming and expensive. However, these couples are considered to be one of the most motivated groups of PGD for aneuploidy screening due to their history of RM and infertility. A clear advantage has been documented in more than 500 clinical cycles with a fourfold reduction in miscarriage rates and an increase of live births (69,70).

Successful FISH involves annealing of the single-stranded fluorescent-labeled DNA probes to its complementary target sequence on a specific chromosome. The biggest limitation to this technique is the fact that only one or two cells are available for analysis. The error rate for single-cell interphase FISH has been recorded in several studies at frequencies between 5 and 15% (18,68,71). Numerous variables could be responsible including signal overlap, signal splitting, cross-hybridization of FISH probes, and the presence of chromosomal mosaicism (72). A greater number of monosomies have been diagnosed by single-cell FISH than trisomies. This could be due to insufficient binding, loss of DNA, poor probe penetration, or an overlap of chromosome signals due to the poor spread of the nucleus during fixation (68). In an attempt to counteract the possibility of misdiagnosis, a FISH scoring system has been implemented (68) to reduce the incidence of false-positive and false-negative results. Nevertheless, several misdiagnoses have been recorded, where aneuploid embryos were misdiagnosed as normal, but on transfer resulted in aneuploid pregnancies that either spontaneously aborted or were detected after prenatal diagnosis (16).

Interestingly, the data from FISH analysis of human IVF embryos have revealed a high incidence of chromosomal mosaicism, with over 30% containing a proportion of aneuploid cells (61,73,74). These high rates of

chromosomal mosaicism observed in human IVF embryos are a major concern in chromosomal aneuploidy screening, questioning the validity of the test, with the possibility of transferring affected mosaic embryos (75,76). Embryonic chromosomal mosaicism is the existence of two or more different chromosomal complements in a single embryo. Mitotic cell division errors post-fertilization, appear to be responsible for the observed chromosomal mosaicism in early human preimplantation development (77,78). The chances of detecting mosaicism would depend on the timing of the mitotic cell division error, e.g., a non-disjunction event during the second cleavage division would result in a 25% chance of biopsing an aneuploid blastomere. Studies have also revealed frequent mosaicism in both the trophectoderm and ICM of human blastocysts (79–81), with the significance at this stage of human embryonic development still to be clarified. The current understanding of the normal dynamics and regulation of mitotic chromosomal segregation during early embryonic cleavage divisions is critically insufficient. Hence, several clinics support the biopsy and analysis of two blastomeres for chromosomal aneuploidy screening in order to reduce the chance of misdiagnosis due to mosaicism (22,82). However, this approach cannot completely overcome the possibility of a misdiagnosis, nor does it address the problem of mosaicism in human IVF embryos. One approach to gaining a better understanding of chromosomal mosaicism during human preimplantation development is to determine the underlying mechanisms causing this phenomenon including the origin and nature of the cell division errors (83). Such knowledge may translate into a revision of current clinical and lab procedures to produce higher numbers of non-mosaic embryos available for transfer, thereby potentially improving implantation rates.

Current single-cell interphase FISH methods are limited to the analysis of less than half of the human chromosomal complement. It is hypothesized that the development of a technique that can analyze all 23 pairs of human chromosomes will allow for the selection of entirely euploid embryos for transfer, further improving pregnancy rates and decreasing miscarriage rates for indicated couples. There are several alternatives that are currently being investigated including metaphase nuclear conversion. Customarily, cytogenetic techniques are performed on cells that are in metaphase, when the nuclear membrane has broken down and the chromosomes are condensed allowing for the identification of each individual chromosome. However, blastomeres are typically observed to be in interphase, when chromosomes are in an unrecognizable state. Metaphase nuclear conversion is a technique that fuses blastomeres or second polar bodies with enucleated or intact oocytes (mouse, bovine, or human) allowing for the metaphase visualization of all 23 pairs of chromosomes to identify both chromosomal aneuploidy and rearrangements. Several methods can then be used on these metaphase converted chromosomes for enumeration including G-banding, chromosome painting, and spectral imaging (84–86).

Metaphase nuclear conversion has been applied in 52 clinical PGD cases for translocations with an 88% success rate and 38 transfers of chromosomally balanced embryos (87). Nevertheless, there are several concerns in producing these metaphase chromosome spreads from a single cell including labor intensity, technical difficulties, and the ethical considerations in fusing human blastomeres with enucleated oocytes from other species.

Another alternative method for enumerating all 23 pairs of chromosomes is comparative genomic hybridization (CGH) (88). This method involves a test DNA sample (blastomere) and a normal control DNA sample differentially labeled with fluorochromes (red or green) and hybridized simultaneously to a spread of normal metaphase chromosomes. If there is a chromosomal imbalance in the test sample, then a deviation of the 1:1 fluorescence ratio will be observed for the particular chromosome affected indicating aneuploidy. The major drawbacks to this method include the inability to detect ploidy changes or balanced translocations, the extremely lengthy labor-intensive procedure taking up to five days to complete, and a substantial amount of starting DNA template (at least 200 ng) necessitating the requirement of WGA for single cells. Several groups have attempted to overcome these difficulties to develop CGH for aneuploidy screening in PGD (89,90). Clinical experience of CGH has included a study of 20 poor IVF prognosis patients resulting in 14 embryo transfers and three clinical pregnancies after freezing and thawing of biopsied embryos (91). In this study, only 54% of the embryos survived the freezing and thawing with at least 50% of cells intact; therefore, to maximize the potential of CGH and the analysis of all chromosomes, it would appear that cryopreservation should be avoided. The biopsy of polar bodies followed by CGH and a blastocyst transfer has also been clinically reported; however, with this method only the maternal chromosomes are available for analysis (92). In its current state, single-cell CGH is a difficult, highly labor-intensive, and complex technique that is unlikely to be widely accepted in clinical PGD for aneuploidy screening.

A new promising technology that could transform chromosomal aneuploidy screening in PGD merges single-cell CGH with a microarray platform. Array CGH is a powerful technique that allows for genome-wide analysis with high resolution and detection of DNA copy number variations (93). Array CGH provides several advantages over conventional CGH, including substantially increased resolution and the ability to directly correlate chromosomal abnormalities with the genomic sequence. Array CGH has the potential to be a primary screening tool for cancer and genetic disease detecting genomic imbalance in cells. It is anticipated that array CGH will transform the practice of both medical genetics and clinical cytogenetics. Currently there are several hurdles that need to be overcome before this technology can be introduced in PGD, including the length of time required for analysis, production of comprehensive WGA products, greater

sensitivity in detecting single copy number changes, adaptability for the IVF clinical setting, and a reduction in the cost of the microarrays themselves. A novel approach that has recently been developed involves whole human chromosome-specific libraries spotted onto glass slides with a total analysis time of just 30 hours (94). This array CGH format has yet to be tested on human embryonic material, but initial results from aneuploid cell lines were promising (94). Microarray technology, still currently in its infancy, has the future capability of providing extremely detailed information on one single platform including chromosome copy number and DNA sequence variations (95).

In summary, PGD is recognized as a reliable and safe reproductive option available to a range of indicated couples including those at risk of passing on an inherited genetic disorder to their offspring and infertile couples with a high chance of producing chromosomally aneuploid embryos. The future of this field will be driven by patient demand, with the number and types of disorders continually expanding. Research and advancing technology will follow suit and involve improvements to the efficiency and reliability of the procedures as well as advances in the techniques utilized for the molecular analysis of a single cell.

REFERENCES

1. Handyside AH, Kontogianni EH, Hardy K, et al. Pregnancies from biopsied human pre-implantation embryos sexed by Y-specific DNA amplification. Nature 1990; 344:768–770.
2. Verlinsky Y, Ginsberg N, Lifchez A, Valle J, Moise J, Strom CM. Analysis of the first polar body: preconception genetic diagnosis. Hum Reprod 1990; 5:826–829.
3. Kuliev A, Verlinsky Y. Thirteen years experience of preimplantation diagnosis: the fifth international symposium on preimplantation genetics. Reprod Biomed Online 1990; 8:229–235.
4. Anon. Report of the 11th Annual Meeting of International Working Group on Preimplantation Genetics: preimplantation genetic diagnosis—experience of 3000 clinical cycles. Reprod Biomed Online 2001; 3:49–53.
5. Handyside AH, Lesko JG, Tarin JJ, et al. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. N Engl J Med 1992; 327:905–909.
6. Snabes MC, Chong SS, Subramanian SB, et al. Preimplantation single-cell analysis of multiple genetic loci by whole-genome amplification. Proc Natl Acad Sci USA 1994; 91:6181–6185.
7. Munne S, Lee A, Rosenwaks Z, Grifo J, Cohen J. Diagnosis of major chromosome aneuploidies in human preimplantation embryos. Hum Reprod 1993; 8: 2185–2191.
8. Verlinsky Y, Cieslak J, Freidine M, et al. Pregnancies following pre-conception diagnosis of common aneuploidies by fluorescent in situ hybridization. Hum Reprod 1995; 10:1923–1927.

9. Conn CM, Harper JC, Winston RM, Delhanty JD. Infertile couples with Robertsonian translocations: preimplantation genetic analysis of embryos reveals chaotic cleavage divisions. Hum Genet 1998; 102:117–123.
10. Scriven PN, Handyside AH, Mackie Ogilvie C. Chromosome translocations: segregation modes and strategies for preimplantation genetic diagnosis. Prenat Diagn 1998; 18:1437–1449.
11. De Vos A, Van Steirteghem A. Aspects of biopsy procedures prior to preimplantation genetic diagnosis. Prenat Diagn 2001; 21:767–780.
12. Cieslak J, Ivakhnenko V, Wolf G, Sheleg S, Verlinsky Y. Three-dimensional partial zona dissection for preimplantation genetic diagnosis and assisted hatching. Fertil Steril 1999; 71:308–313.
13. Grifo JA, Tang YX, Munné S, et al. Healthy babies from biopsied human embryos. Hum Reprod 1994; 9:912–916.
14. Tadir Y, Neev J, Ho P, Berns MW. Lasers for gamete micromanipulation: basic concepts. J Assist Reprod Genet 1993; 10:121–125.
15. Boada M, Carrera M, De La Iglesia C, et al. Successful use of laser for human embryo biopsy in preimplantation genetic diagnosis: report of two cases. J Assist Reprod Genet 1998; 15:301–306.
16. ESHRE PGD Consortium. Hum Reprod 2002; 20:35–48.
17. Verlinsky Y, Rechitsky S, Cieslak J, et al. Preimplantation diagnosis of single gene disorders by two-step oocyte genetic analysis using first and second polar body. Biochem Mol Med 1997; 62:182–187.
18. Verlinsky Y, Cieslak J, Ivakhnenko V, et al. Implantation diagnosis of common aneuploidies by the first and second-polar body FISH analysis. J Assist Reprod Genet 1998; 15:285–289.
19. Handyside AH, Pattinson JK, Penketh RJ, et al. Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet 1989; 1:347–349.
20. Hardy K, Martin KL, Leese HJ, et al. Human pre-implantation development in vitro is not adversely affected by biopsy at the 8-cell stage. Hum Reprod 1990; 5: 708–714.
21. Tarin JJ, Conaghan J, Winston RM, et al. Human embryo biopsy on the 2nd day after insemination for preimplantation diagnosis: removal of a quarter of embryo retards cleavage. Fertil Steril 1992; 58:970–976.
22. Van de Velde H, De Vos A, Sermon K, et al. Embryo implantation after biopsy of one or two cells from cleavage-stage embryos with a view to preimplantation genetic diagnosis. Prenat Diagn 2000; 20:1030–1037.
23. Emiliani S, Gonzalez-Merino E, Englert Y, Abramowicz M. Comparison of the validity of preimplantation genetic diagnosis for embryo chromosomal anomalies by fluorescence in situ hybridization on one or two blastomeres. Genet Test 2004; 8:69–72.
24. Dokras A, Sargent IL, Ross C, et al. Trophectoderm biopsy in human blastocysts. Hum Reprod 1990; 5:821–825.
25. Veiga A, Sandalinas M, Benkhalifa M, et al. Laser blastocyst biopsy for preimplantation diagnosis in the human. Zygote 1997; 5:351–354.
26. Veiga A, Gil Y, Boada M, et al. Confirmation of diagnosis in preimplantation genetic diagnosis (PGD) through blastocyst culture: preliminary experience. Prenat Diagn 1999; 19:1242–1247.

27. Dokras A, Sargent IL, Gardner RL, et al. Human trophectoderm biopsy and secretion of chorionic gonadotrophin. Hum Reprod 1991; 6:1453–1459.
28. de Boer KA, Catt JW, Jansen RP, Leigh D, McArthur S. Moving to blastocyst biopsy for preimplantation genetic diagnosis and single embryo transfer at Sydney IVF. Fertil Steril 2004; 82:295–298.
29. Kokkali G, Vrettou C, Traeger-Synodinos J, et al. Birth of a healthy infant following trophectoderm biopsy from blastocysts for PGD of beta-thalassaemia major: case report. Hum Reprod 2005; 20:1855–1859.
30. Wells D, Delhanty JD. Preimplantation genetic diagnosis: applications for molecular medicine. Trend Mol Med 2001; 7:23–30.
31. Verlinsky Y, Cohen J, Munne S, et al. Over a decade of experience with preimplantation genetic diagnosis: a multicenter report. Fertil Steril 2004; 82:292–294.
32. Morton NE. Parameters of the human genome. Proc Natl Acad Sci USA 1991; 88:7474–7476.
33. Erlich HA, Arnheim N. Genetic analysis using the polymerase chain reaction. Ann Rev Genet 1992; 26:479–506.
34. Kuliev A, Rechitsky S, Verlinsky O, et al. Preimplantation diagnosis of thalassemias. J Assist Reprod Genet 1998; 15:219–225.
35. Strom CM, Rechitsky S, Wolf G, et al. Preimplantation diagnosis of autosomal dominant retinitis pigmentosum using two simultaneous single cell assays for a point mutation in the rhodopsin gene. Mol Hum Reprod 1998; 4:351–355.
36. Larsen LA, Christiansen M, Vuust J, et al. High-throughput single-strand conformation polymorphism analysis by automated capillary electrophoresis: robust multiplex analysis and pattern-based identification of allelic variants. Hum Mut 1999; 13:318–327.
37. Vrettou C, Palmer G, Kanavakis E, et al. A widely applicable strategy for single cell genotyping of β-thalassaemia mutations using DGGE analysis: application to preimplantation genetic diagnosis. Prenat Diagn 1999; 9:1209–1216.
38. Ao A, Wells D, Handyside AH, et al. Preimplantation genetic diagnosis of inherited cancer familial adenomatous polyposis coli. J Assist Reprod Genet 1998; 15:140–144.
39. Cram DS, Song B, Trounson AO. Preimplantation diagnosis of Lesch–Nyhan using mini-sequencing primer extension. Reprod Biomed Online 2003; 7: 342–345.
40. Sermon K, De Vos A, Van de Velde H, et al. Fluorescent PCR and automated fragment analysis for the clinical application of preimplantation genetic diagnosis of myotonic dystrophy (Steinert's disease). Mol Hum Reprod 1998; 4: 791–796.
41. Sermon K, Goossens V, Seneca S, et al. Preimplantation diagnosis for Huntington's Disease (HD): clinical application and analysis of the HD expansion in affected embryos. Prenat Diagn 1998; 18:1427–1436.
42. Pals G, Young C, Mao HS, et al. Detection of a single base substitution in a single cell using the LightCycler. J Biochem Biophys Meth 2001; 47:121–129.
43. Vrettou C, Traeger-Synodinos J, Tzetis M, Palmer G, Sofocleous C, Kanavakis E. Real-time PCR for single-cell genotyping in sickle cell and thalassemia syndromes as a rapid, accurate, reliable, and widely applicable protocol for preimplantation genetic diagnosis. Hum Mutat 2004; 23:513–521.

44. Thornhill AR, deDie-Smulders CE, Geraedts JP, et al. ESHRE PGD Consortium on 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS).' Hum Reprod 2005; 20: 35–48.
45. Rechitsky S, Verlinsky O, Amet T, et al. Reliability of preimplantation diagnosis for single gene disorders. Mol Cell Endocrinol 2001; 183(suppl 1):S65–S68.
46. Jeffreys AJ, Wilson V, Thein SL. Individual-specific 'fingerprints' of human DNA. Nature 1985; 316:76–78.
47. Katz MG, Trounson AO, Cram DS. DNA fingerprinting of sister blastomeres from human IVF embryos. Hum Reprod 2002; 17:752–759.
48. Hardy K, Winston RM, Handyside AH. Binucleate blastomeres in preimplantation human embryos in vitro: failure of cytokinesis during early cleavage. J Reprod Fertil 1993; 98:549–558.
49. Ray PF, Handyside AH. Increasing the denaturation temperature during the first cycles of amplification reduces allele dropout from single cells for preimplantation genetic diagnosis. Mol Hum Reprod 1996; 2:213–218.
50. Lewis CM, Pinel T, Whittaker JC, et al. Controlling misdiagnosis errors in preimplantation genetic diagnosis: a comprehensive model encompassing extrinsic and intrinsic sources of error. Hum Reprod 2001; 16:43–50.
51. Piyamongkol W, Bermudez MG, Harper JC, et al. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. Mol Hum Reprod 2003; 9:411–420.
52. Salvado CS, Trounson AO, Cram DS. Towards preimplantation diagnosis of cystic fibrosis using microarrays. Reprod Biomed Online 2004; 8:107–114.
53. Paunio T, Reima I, Syvanen AC. Preimplantation diagnosis by whole genome amplification, PCR amplification and solid-phase minisequencing of blastomere DNA. Clin Chem 1996; 42:1382–1390.
54. Sermon K, Lissens W, Joris H, et al. Adaptation of the primer extension preamplification (PEP) reaction for preimplantation diagnosis: single blastomere analysis using short PEP protocols. Mol Hum Reprod 1996; 2:209–212.
55. Zhang L, Cui X, Schmitt K, et al. Whole genome amplification from a single cell: implications for genetic analysis. Proc Natl Acad Sci USA 1992; 89:5847–5851.
56. Cheung VG, Nelson SF. Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. Proc Natl Acad Sci USA 1996; 93:14676–14679.
57. Dean FB, Hosono S, Fang L, et al. Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA 2002; 99: 5261–5266.
58. Hellani A, Coskun S, Tbakhi A, Al-Hassan S. Clinical application of multiple displacement amplification in preimplantation genetic diagnosis. Reprod Biomed Online 2005; 10:376–380.
59. Handyside A, Robinson MD, Sinpson RJ, et al. Isothermal whole genome amplification from single and small numbers of cells: a new era for preimplantation genetic diagnosis of inherited disease. Mol Hum Reprod 2004; 10:767–772.
60. Sun G, Kaushal R, Pal P, et al. Whole-genome amplification: relative efficiencies of the current methods. Leg Med (Tokyo) 2005; 7:279–286.

61. Delhanty JD, Griffin DK, Handyside AH, et al. Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent in situ hybridisation (FISH). Hum Mol Genet 1993; 2: 1183–1185.
62. Gianaroli L, Magli MC, Ferraretti AP, et al. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with a poor prognosis: identification of the categories for which it should be proposed. Fertil Steril 1999; 72:837–844.
63. Munné S, Sandalinas M, Escudero T, et al. Improved implantation after preimplantation genetic diagnosis of aneuploidy. Reprod Biomed Online 2003; 7: 91–97.
64. Boue A, Boue J, Gropp A. Cytogenetics of pregnancy wastage. Adv Hum Genet 1985; 14:1–57.
65. Hook EB. Chromosome abnormalities. In: Brock DJH, Rodek CH, Ferguson-Smith MA, eds. Prenatal Diagnosis and Screening. Edinburgh: Churchill Livingstone, 1992.
66. Verlinsky Y, Cohen J, Munne S, et al. Over a decade of experience with preimplantation genetic diagnosis. Fertil Steril 2004; 82:302–303.
67. Munne S, Chen S, Fischer J, et al. Preimplantation genetic diagnosis reduces pregnancy loss in women aged 35 years and older with a history of recurrent miscarriages. Fertil Steril 2005; 84:331–335.
68. Munné S, Magli C, Bahce M, et al. Preimplantation diagnosis of the aneuploidies most commonly found in spontaneous abortions and live births: XY, 13, 14, 15, 16, 18, 21, 22. Prenat Diagn 1998; 18:1459–1466.
69. Gianaroli L, Magli MC, Ferraretti AP, et al. Possible interchromosomal effect in embryos generated by gametes from translocation carriers. Hum Reprod 2002; 17:3201–3207.
70. Verlinsky Y, Cieslak J, Evsikov S, et al. Nuclear transfer for full karyotyping and preimplantation diagnosis for translocations. Reprod Biomed Online 2002; 5: 300–305.
71. Magli MC, Sandalinas M, Escudero T, et al. Double locus analysis of chromosome 21 for preimplantation genetic diagnosis of aneuploidy. Prenat Diagn 2001; 21:1080–1085.
72. Connen E, Hopman AHN, Geraedts JPM, et al. Application of in situ hybridization techniques to study human preimplantation embryos: a review. Hum Reprod Update 1998; 4:135–152.
73. Munné S, Weier H, Grifo J, et al. Chromosome mosaicism in human embryos. Biol Reprod 1994; 51:373–379.
74. Harper JC, Coonen E, Handyside AH, et al. Mosaicism of autosomes and sex chromosomes in morphologically normal monospermic preimplantation human embryos. Prenat Diagn 1995; 15:41–49.
75. Kuo HC, Ogilvie CM, Handyside AH. Chromosomal mosaicism in cleavage-stage human embryos and the accuracy of single-cell genetic analysis. J Assist Reprod Genet 1998; 15:276–280.
76. Magli MC, Sandalinas M, Escudero T, et al. Double locus analysis of chromosome 21 for preimplantation genetic diagnosis of aneuploidy. Prenat Diagn 2001; 21:1080–1085.

77. Coonen E, Derhaag JG, Dumoulin J, et al. Anaphase lagging mainly explains chromosomal mosaicism in human preimplantation embryos. Hum Reprod 2004; 19:316–324.
78. Katz-Jaffe MG, Trounson AO, Cram DS. Mitotic errors in chromosome 21 of human pre-implantation embryos are associated with non-viability. Mol Hum Reprod 2004; 10:143–147.
79. Evsikov S, Verlinsky Y. Mosaicism in the inner cell mass of the human blastocysts. Hum Reprod 1998; 13:3151–3155.
80. Ruangvutilert P, Delhanty JD, Serhal P, et al. FISH analysis on day 5 post-insemination of human arrested and blastocyst stage embryos. Prenat Diagn 2000; 20:552–560.
81. Bielanska M, Jin S, Bernier M, Tan SL, Ao A. Diploid–aneuploid mosaicism in human embryos cultured to the blastocyst stage. Fertil Steril 2005; 84:336–342.
82. Simopoulou M, Harper JC, Fragouli E, et al. Preimplantation genetic diagnosis of chromosome abnormalities: implications from the outcome for couples with chromosomal rearrangements. Prenat Diagn 2003; 23:652–662.
83. Katz-Jaffe MG, Trounson AO, Cram DS. Chromosome 21 mosaic human preimplantation embryos predominantly arise from diploid conceptions. Fertil Steril 2005; 84:634–643.
84. Verlinsky Y, Evsikov S. A simplified and efficient method for obtaining metaphase chromosomes from individual human blastomeres. Fertil Steril 1999; 72:1127–1133.
85. Willadsen S, Levron J, Munné S, et al. Rapid visualization of metaphase chromosomes in single human blastomeres after fusion with in vitro matured bovine eggs. Hum Reprod 1999; 14:470–475.
86. Eviskov S, Cieslak J, Verlinsky Y. Effect of chromosomal translocations on the development of preimplantation human embryos in vitro. Fertil Steril 2000; 74:672–677.
87. Verlinsky Y, Cieslak J, Evsikov S, et al. Nuclear transfer for full karyotyping and preimplantation diagnosis for translocations. Reprod Biomed Online 2002; 5:300–305.
88. Kallioniemi A, Kallioniemi OP, Sudar D, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992; 258: 818–821.
89. Voullaire L, Slater H, Williamson R, et al. Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. Hum Genet 2000; 106:210–217.
90. Wells D, Delhanty JD. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. Mol Hum Reprod 2000; 6:1055–1062.
91. Wilton L, Voullaire L, Sargeant P, et al. Preimplantation aneuploidy screening using comparative genomic hybridization or fluorescence in situ hybridization of embryos from patients with recurrent implantation failure. Fertil Steril 2003; 80:860–868.
92. Wells D, Escudero T, Levy B, et al. First clinical application of comparative genomic hybridization and polar body testing for preimplantation genetic diagnosis of aneuploidy. Fertil Steril 2002; 78:543–549.

93. Pinkel D, Segraves R, Sudar D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998; 20:207–211.
94. Hu DG, Webb G, Hussey N. Aneuploidy detection in single cells using DNA array-based comparative genomic hybridization. Mol Hum Reprod 2004; 10: 283–289.
95. Yi Y, Mirosevich J, Shyr Y, Matusik R, George AL Jr. Coupled analysis of gene expression and chromosomal location. Genomics 2005; 85:401–412.

16

Oocyte and Embryo Cryopreservation

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HISTORY OF MAMMALIAN GAMETE/EMBRYO CRYOBIOLOGY

When one considers the history of gamete/embryo cryobiology, it is difficult to select a specific event and point of origin. Early basic scientific advancements in measurements of temperature and the chemistry of solutions and gases are certainly sentinel events for cryobiology. It has been suggested that original versions of a device to measure temperature were made by Galileo Galilei in the early seventeenth century. The first accurate means of measuring temperature were developed in the early 1700s by the German physicist Gabriel Fahrenheit through application of mercury in glass. Since these early days, modifications of instruments to assess temperature have become significantly more accurate and easier to use. Equally important were early advancements made in the nineteenth century involving understandings of liquefaction of gases and potential use of such refrigerants to cool and store specimens at extremely low temperatures.

When one traces the history of mammalian gamete cryopreservation, numerous accounts reference the beginning of low-temperature biology to 1866, when an Italian military physician Mantegazza documented the observation that human spermatozoa became immotile when cooled in snow

(1,2). He subsequently proposed that it might be possible for a soldier to father a child, even after his death, by cooling and storing spermatozoa. It is quite interesting that centuries later, as technological refinement of cryopreservation has occurred, many of these same reproductive quandaries exist and are still debated today (3–5). In the early 1930s, two papers were published (6,7) that demonstrated the effects of numerous temperatures ranging from 0 to 45°C on rabbit spermatozoa. Considering advances that were being made at this time in basic understandings of reproductive biology, artificial insemination, and aeronautical engineering, Dr. Hammond demonstrated his forward-thinking by stating "in these days of rapid aeroplane transport, it might be possible to move entire herds of animals around the world in the form of chilled samples of semen" (1). Although there were numerous empirical studies on non-mammalian and mammalian spermatozoa cryopreservation in the 1930s and 1940s, a major breakthrough occurred with the serendipitous discovery that glycerol imparts cryoprotective properties to spermatozoa during freezing and thawing (8). A tantalizing tale of this important moment in cryobiological history is eloquently portrayed by Leibo (1). In the early 1950s, Bunge and Sherman (9,10) extended Polge's use of glycerol to cryopreservation of human sperm. This ability to cryopreserve human sperm that upon thawing can fertilize ova has subsequently resulted in thousands, and perhaps millions, of children being born through intrauterine insemination (IUI) of cryopreserved semen.

Investigations in the late 1940s and early 1950s by Chang (11,12) on low-temperature storage of rabbit oocytes, zygotes, and embryos paved the way for studies on the cryopreservation of female gametes and embryos. Subsequent experiments by Sherman and Lin (13–16) demonstrated that mouse oocytes could also be cooled in glycerol, stored and subsequently fertilized in recipients; furthermore, resulting embryos supported pregnancies. In the 1960s and early 1970s, a merging of basic/theoretical cryobiology and practical studies ultimately gave rise to increased success of embryo cryopreservation. Classical basic science investigations by Mazur (17–19) formed the foundation for understanding cell-specific optimal cooling and warming rates which today remain a pivotal key to successful mammalian gamete and embryo cryopreservation. It was the combined strengths of Mazur, Leibo, and Whittingham that resulted in successful cryopreservation of mouse embryos (Fig. 1). These investigators used 1.5 M dimethylsulfoxide (DMSO) as the cryoprotective agent combined with a slow cooling rate (−0.3°C/min to −80°C) and stored in liquid nitrogen (20). This procedure was based on the appreciation that slow cooling rates would support dehydration during cooling and avoid intracellular ice formation. In addition, it was established that addition and removal of the cryoprotective agent (here DMSO) should be performed in a stepwise manner to avoid osmotic shock or damage.

In the early 1980s, application of the same methodologies of cryopreservation led to the establishment of the first human pregnancies following

Figure 1 A photograph of Mazur (*left*), Leibo (*center*), and Whittingham (*right*) taken in June 1972 on the occasion of the birth of the first mammals derived from cryopreserved embryos.

freezing and thawing (21,22). The use of 1,2-propanediol (PROH) as a permeating cryoprotectant for pronuclear-stage zygotes was introduced by Testart et al. (23). In addition, these investigators used sucrose in the cryo-media as a non-permeating cryoprotectant to act as an osmotic buffer. Slow cooling rates were used until −30°C was reached; samples were subsequently plunged into liquid nitrogen, and the warming rate was rapid. This approach of cryopreserving pronuclear- and cleavage-stage mammalian embryos has become an acceptable procedure in assisted reproductive technology (ART) laboratories across the United States and worldwide.

PRINCIPLES OF CRYOPRESERVATION

As mentioned previously, in the 1940s it was discovered that addition of glycerol protected against cryo-damage and greatly enhanced survival of cryopreserved living cells. This led to the investigational concept of cryoprotectants. In hindsight, the use of cryoprotectants is logical considering that, in insect biological systems, sugars and sugar-alcohols are used to withstand severe winter temperatures (24). Experience in cryopreservation of various cell types led to the appreciation that as cell size increases, difficulty in cryopreservation also increases (19). This concept is of particular importance in mammalian oocyte and embryo cryopreservation.

Currently, there are two methods used to cryopreserve mammalian oocytes and embryos: slow-rate freezing and vitrification (25). Independent of the methodology used for cryopreservation, effects on oocyte and embryonic cellular functions can compromise abilities to develop normally following the cryopreservation process. These compromised cellular events

can be collectively termed oocyte and/or embryo "cryo-damage." Documented and/or theoretical-specific cellular structures and functions that are/may be compromised by cryopreservation, as well as subsequent effects on oocyte and embryonic developmental competence have been previously reviewed (26).

During cryopreservation, cells are exposed to numerous stresses including mechanical, thermal, and chemical (19,27), which can lead to compromised cell function and cell death. In general, it has been demonstrated that oocytes are more sensitive to cryo-damage than later embryonic stages (28). A detailed discussion of the biophysics of cryopreservation is beyond the scope of this review, yet such information is available (18). Slow-rate freezing attempts to control biophysical properties of freezing, such as cooling and warming rates, in conjunction with cryoprotectants to minimize adverse cellular events. This method allows cells to be cooled to very low temperatures while minimizing intracellular ice crystal formation, and at the same time attempting to minimize the detrimental influences of increased solute concentrations and osmotic stress (28). Thus one can appreciate that with slow-rate freezing extracellular ice formation drives cellular dehydration through an equilibrium process. Conversely, vitrification, a form of rapid cooling, utilizes very high concentrations of cryoprotectant that solidify without forming ice crystals. The term "vitrification" is derived from the Latin word *vitreous*, which means glassy or resembling glass. Vitrification can be considered a non-equilibrium approach to cryopreservation originally developed for cryopreservation of mammalian sperm (8) and embryos (29). The vitrified solids therefore contain the normal molecular and ionic distributions of the original liquid state and can be considered an extremely viscous, supercooled liquid (30). In this technique, oocytes or embryos are dehydrated by brief exposure to a concentrated solution of cryoprotectant before plunging the samples directly into liquid nitrogen. Utilization of vitrification for both oocytes and embryos is an area of current focus for many clinical, rodent, and domestic animal production laboratories. Human oocytes (31), pronuclear zygotes (32), cleavage-stage embryos (33), and blastocysts (34–36) have been successfully vitrified. Excellent reviews of vitrification history, utilization, and potential advantages are available (37,38).

PROTOCOLS

It is important to recognize that there are important fundamental aspects of gamete/embryo cryopreservation that must be followed for successful cell survival, normal cellular function, and subsequent development. It is important also to recognize that numerous slight permutations exist between cryopreservation protocols that do not appear to influence clinical outcomes. This generalizing statement, while non-scientific and potentially concerning,

is a result of inabilities to perform well-controlled, randomized, prospective clinical trials with human oocytes/embryos. Thus, keys to successful gamete/embryo cryopreservation are basing protocols on a combined strong understanding of cell developmental biology, empirical and theoretical aspects of cryobiology, and practical experience. Below are protocols for both slow-rate freezing/thawing and vitrification/warming that are known to work well for oocytes, cleavage-stage embryos, and blastocysts. Obviously, there are numerous protocols that vary slightly from those described below, and are known to provide commendable results.

Slow-Rate Freezing

Oocytes

Numerous reports of successful human oocyte slow-rate freezing and thawing with subsequent healthy live births exist (Table 1). The following is a human oocyte slow-rate freezing protocol based on work from Porcu et al. (52).

Freezing:

1. Materials (*expendables*): Conical tubes, organ culture dish, 5-and 10-mL disposable pipettes, pulled-pipettes—inner diameter just larger than the oocytes being cryopreserved, 0.2 μM filter, cryo-straws, cryo-canes, cryo-goblets, and cryo-sleeves.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, heat-sealer, programmable biological freezer, liquid nitrogen storage canister with lock and alarm, safety goggles, and cryo-gloves.
3. Cryo-solutions:
   Freeze medium #1 = phosphate-buffered saline (PBS) + 18% (w/v) protein source (WASH)
   Freeze medium #2 = freeze medium #1 + 1.5 M 1,2-PROH
   Freeze medium #3 = freeze medium #2 + 0.3 M sucrose (PROH + SUCR)
4. Procedure
   a. Prepare freeze media inside the hood, at least one day prior to use. Rinse all tubes, syringes, and filters with PBS prior to use. Filter all freeze media with a 0.2-μM syringe filter.
   b. Label three organ culture dishes with patient identifiers and one each with WASH, PROH, PROH + SUCR. After rinsing dishes, add 1 mL of the appropriate medium to each dish and keep at room temperature.
   c. Two to three hours after egg retrieval, denude oocytes and select MII oocytes for freezing.

Table 1 Live Births from Slow-Rate Frozen Human Oocytes (Empty Cells Indicate Data Not Available)

Authors	Year	Method		Survival (%)	Fertilization (%)	Infants born (#)
		Cryoprotectants	Device			
Chen (39)	1986	DMSO		80	83	2
Van Uem et al. (40)	1987	DMSO	Straws	25	50	1
Porcu et al. (41,42)	1997, 1998	PROH, sucrose	Straws	33	50	1
				56	63	6
Tucker et al. (43)	1998	PROH, sucrose	Glass ampoules	24	51	3
				13	50	1
Polak de Fried (44)	1998	PROH, sucrose	Straws	30	66	1
Porcu et al. (45)	2000	PROH, sucrose	Straws	55–59	57–64	12
Winslow et al. (46)	2001	PROH, sucrose		68	81	16
Quintans et al. (47)	2002	PROH, sucrose	Straws	63	59	2
Yang et al. (48)	2002	PROH, sucrose		71	86	14
Boldt et al (49)	2003	PROH, sucrose low Na^+choline	Nunc vials	74	59	5
Fosas et al. (50)	2003	PROH, sucrose	Straws	90	73	5
Borini et al. (51)	2004	PROH, sucrose	Straws	37	45	13
Total documented live births from these citations						77

Abbreviations: DMSO, dimethylsulfoxide; PROH, 1,2-propanediol.

d. Transfer oocytes for freezing into WASH.

- When transferring oocytes to a dish, always aspirate a small volume of the medium into which you are moving the oocytes prior to picking them up.
- Move the oocytes in as small a volume of medium as possible.
- Place the oocytes in at least three different areas of the dish to fully rinse them of the last medium.

e. Transfer oocytes into PROH for 10 minutes.
f. Transfer oocytes in PROH + SUCR for five minutes. During that five minutes period:

- Label the straw(s), canes(s), goblet(s), and or sleeve(s) with patient identifiers.
- Rinse the straw(s) with PROH + SUCR and expel (do not let medium touch the cotton-plug/sealant end).
- Load the straw(s) with PROH + SUCR and oocytes in the following order:

  a. 1-inch medium
  b. 1/4-inch air space
  c. 1/2-inch medium
  d. 1-inch medium containing oocytes
  e. 1/4-inch air space
  f. Fill the remainder of the straw with medium. Make sure the medium completely wets the straw sealant at the end of the straw to ensure a proper seal.

- Seal the straw(s)
- Place straw(s) into programmable biological freezer with the sealant plug toward the top.

g. Once straw(s) are loaded into the programmable freezer, begin the program.

Rate (°C/min)	Target temperature (°C)	Hold time (min)
0.0	20.0	5.0
2.0	−7.0	10
0.3	−30.0	0.0
50	−150	0.0

h. When the temperature changes to −7°C, hold for five minutes, then seed straw(s) by briefly touching a liquid-nitrogen-soaked cotton-tipped applicator at the meniscus just below the first airspace.
i. Wait two minutes, then inspect the seeded straw(s) to confirm ice crystal formation/growth.
j. When the programmable biological freezer temperature reaches −30°C, it can initiate free-fall to −150°C. Samples can then be removed and plunged into liquid nitrogen after ten to twelve minutes of stabilization at this temperature. During transfer, use goggles, cryo-protective gloves, and liquid-nitrogen-cooled forceps. Place straw(s) into liquid-nitrogen-submerged goblet on cane, and rapidly transfer into liquid nitrogen storage canister.

Thawing:

1. Materials (*expendables*): Conical tubes, organ culture dish, 5 and 10 mL disposable pipettes, pulled-pipettes—inner diameter just larger than the oocytes being cryopreserved, 0.2 μM filter, and paper tissues.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, Dewar, timer, scissors, straw plunger, safety goggles and cryo-gloves, incubators (CO_2 and non-CO_2, 37°C).
3. Thaw-solutions:
   Thaw medium #1 = PBS + 18% (w/v) protein source (TM1 or WASH)
   Thaw medium #2 = TM1 + 0.3 M sucrose (SUCR)
   Thaw medium #3 = TM1 + 1.0 M PROH + 0.3 M sucrose (1.0 M PROH + SUCR)
   Thaw medium #4 = TM1 + 0.5 M PROH + 0.3 M sucrose (0.5 M PROH + SUCR)

4. Procedure
   a. Prepare thaw media inside the hood, at least one day prior to use. Rinse all tubes, syringes, and filters with PBS prior to use. Filter all thaw media with a 0.2 μM syringe filter.
   b. Prepare oocyte culture dishes and place into CO_2 incubator at least four hours before thaw.
   c. Label five organ culture dishes with patient identifiers and one each with 1.0 M PROH + SUCR, 0.5 M PROP + SUCR, SUCR, WASH1, WASH2. After rinsing dishes, add 1.0 mL of the appropriate medium to each dish and keep at room temperature (except for WASH2–37°C).

d. Transfer the cryo-straw(s) containing oocytes to be thawed from the liquid nitrogen storage canister to a filled liquid-nitrogen-portable Dewar. Always wear protective goggles and gloves when handling liquid nitrogen. Check the cryo-straw out of the patient oocyte logbook and out of the patient's cryopreservation record. Transport the cryo-straw(s) in liquid nitrogen to the site of thaw.
e. Remove the straw from the liquid-nitrogen-filled Dewar with forceps. Hold the straw horizontally at the sealed, cotton-plugged end, and allow the straw to partially thaw at room temperature for 30 seconds.
f. Gently wipe the straw to remove condensation.
g. Submerge the straw in 30°C water bath for 40 seconds.
h. Remove the straw from the water bath and wipe dry.
i. Expel the oocytes from the straw.
   - Hold the straw vertically over the organ culture dish containing 1.0 M PROH + SUCR with the sealed, cotton-plug end up.
   - Cut the sealed end of the straw, opposite to the cotton-plug end.
   - Cut the cotton-plug end in the middle of the seal.
   - Use a straw plunger to expel the contents of the straw into the organ culture dish containing 1.0 M PROH + SUCR.
   - Move the oocytes to three different areas of the dish and hold for five minutes.
j. Transfer oocytes to 0.5 M PROH + SUCR for five minutes.
   - When transferring oocytes to another dish always aspirate, a small volume of the medium into which you are moving oocytes prior to picking them up.
   - Move oocytes in as small of a volume of medium as possible.
   - Place oocytes in at least three different areas of the dish to fully rinse them of the last medium.
k. Transfer oocytes to SUCR and leave oocytes in this solution for 10 minutes.
l. Transfer oocytes to WASH1 and leave oocytes in this solution for 10 minutes.
m. Transfer oocytes to WASH2 (at 37°C) and leave oocytes in this solution for 10 minutes.
n. Transfer oocytes into oocyte-culture dish (bicarbonate-buffered media + protein) and place into CO_2 incubator for four hours before proceeding with intracytoplasmic sperm injection.

Cleavage-Stage Embryos

Cleavage-stage embryos can be frozen successfully on either day 2 or 3 (d0 = day of insemination). It is important to note that embryos can be cryopreserved in straws or vials. The below protocol is based on use of straws as cryo-containers.

Freezing:

1. Materials (*expendables*): Conical tubes, organ culture dish, 5- and 10-mL disposable pipettes, pulled-pipettes—inner diameter just larger than the embryos being cryopreserved, 0.2 μm filter, cryo-straws, cryo-canes, cryo-goblets, and cryo-sleeves.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, heat-sealer, programmable biological freezer, liquid nitrogen storage canister with lock and alarm, safety goggles, and cryo-gloves.
3. Cryo-solutions:
   Freeze medium #1 = HEPES [4(2-hydroxyethyl)1-piperazineethane sulfonic acid)]-buffered medium + 12–15% (w/v) protein source (WASH)
   Freeze medium #2 = freeze medium #1 + 1.5 M 1,2-PROH
   Freeze medium #3 = freeze medium #2 + 0.1 M sucrose (PROH + SUCR)
4. Procedure
   a. Prepare freeze media inside the hood, at least one day prior to use. Rinse all tubes, syringes, and filters with HEPES-buffered media prior to use. Filter all freeze media with a 0.2 μM syringe filter.
   b. Label four organ culture dishes with patient identifiers and one each with WASH1, WASH2, PROH, PROH + SUCR. After rinsing dishes, add 1 mL of the appropriate medium to each dish and keep at room temperature.
   c. Select embryos to freeze that meet your specific freezing criteria.
   d. Transfer embryos to freeze into WASH1.
      - When transferring embryos to a dish, always aspirate a small volume of the medium into which you are moving the embryos prior to picking them up.
      - Move the embryos in as small of a volume of medium as possible.
      - Place the embryos in at least three different areas of the dish to fully rinse them of the last medium.
   e. Transfer embryos into WASH2 dish.
   f. Transfer embryos into PROH for 15 minutes.

g. Transfer embryos into PROH + SUCR for 15 minutes. During that 15-minute period:

- Label the straw(s), canes(s), goblet(s), and or sleeve(s) with the patient identifiers.
- Rinse the straw(s) with PROH + SUCR and expel (do not let medium touch pre-sealed end).
- Load the straw(s) with PROH + SUCR and embryos in the following order:

    a. 1-inch medium
    b. 1/4-inch air space
    c. 1/2-inch medium
    d. 1-inch medium containing embryos
    e. 1/4-in air space
    f. Fill the remainder of the straw with medium. Make sure the medium completely wets the pre-sealed end of the straw to ensure a proper seal.

- Seal the straw(s)
- Place straw(s) into programmable biological freeze.

h. Once straw(s) are loaded into the programmable freezer, begin the program. The program listed below is one of many that has been successfully used to cryopreserve embryos.

Rate (°C/min)	Target temperature (°C)	Hold time (min)
0.0	20.0	5.0
2.0	−5 to −7	15.0
0.3	−32.0	0.0

i. When the temperature changes to −5 to −7 (seeding temperature), hold for five minutes, then seed straw(s) by briefly touching a liquid-nitrogen-soaked cotton-tipped applicator at the meniscus just below the first airspace.
j. Wait two minutes; inspect the seeded straw(s) to confirm ice crystal formation/growth.
k. When the programmable biological freezer temperature reaches −32°C, the programmable biological freezer can initiate free-fall or samples can be removed and plunged into liquid nitrogen. During transfer, use goggles, cryo-protective gloves, and liquid-nitrogen-cooled forceps. Place straw(s) into liquid-nitrogen-submerged goblet on cane, and rapidly transfer into liquid nitrogen storage canister.

Thawing:

1. Materials (*expendables*): Conical tubes, organ culture dish, 5- and 10-mL disposable pipettes, puller-pipettes—inner diameter just larger then the embryos being cryopreserved, 0.2 μm filter, paper tissues.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, Dewar, timer, scissors, straw plunger, safety goggles and cryo-gloves.
3. Thaw-solutions:
   Thaw medium #1 = HEPES-buffered medium + 12–15% (w/v) protein source (TM1 or WASH)
   Thaw medium #2 = TM1 + 0.2 M sucrose (SUCR)
   Thaw medium #3 = TM1 + 1.0 M PROH + 0.2 M sucrose (1.0 M PROH + SUCR)
   Thaw medium #4 = TM1 + 0.5 M PROH + 0.2 M sucrose (0.5 M PROH + SUCR)

4. Procedure
   a. Prepare thaw media inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters with HEPES-buffered medium prior to use. Filter all thaw media with a 0.2 μM syringe filter.
   b. Prepare embryo culture dishes and place into CO_2 incubator at least four hours before thaw.
   c. Label five organ culture dishes with the patient identifiers and one each with 1.0 M PROH + SUCR, 0.5 M PROH + SUCR, SUCR, WASH1, WASH2. After rinsing dishes, add 1.0 mL of the appropriate medium to each dish and keep at room temperature.
   d. Transfer the cryo-straw(s) containing embryos to be thawed from the liquid nitrogen storage canister to a filled liquid-nitrogen-portable Dewar. Always wear protective goggles and gloves when handling liquid nitrogen. Check the cryo-straw out of the patient embryo logbook and out of the patient's cryopreservation record. Transport the cryo-straw(s) in liquid nitrogen to the site of thaw.
   e. Remove the straw from the liquid-nitrogen-filled Dewar with forceps. Hold the straw horizontally at the sealed, cotton-plugged end and allow the straw to partially thaw at room temperature for 40 seconds.
   f. Gently rub the straw between your fingers with a paper tissue until all the ice crystals have disappeared.
   g. Expel the embryos from the straw.

- Hold the straw vertically over the organ culture dish containing 1.0 M PROH + SUCR with the sealed, cotton-plug end up.
- Cut the sealed end of the straw, opposite to the cotton-plug end.
- Cut the cotton-plug end in the middle of the seal.
- Use a straw plunger to expel the contents of the straw into the organ culture dish containing 1.0 M PROH + SUCR.
- Move the embryos to three different areas of the dish and hold for five minutes.

h. Transfer embryos to 0.5 M PROH + SUCR for five minutes.
   - When transferring embryos to another dish, always aspirate a small volume of the medium into which you are moving embryos prior to picking them up.
   - Move embryos in as small of a volume of medium as possible.
   - Place embryos in at least three different areas of the dish to fully rinse them of the last medium.

i. Transfer embryos to SUCR for five minutes.
j. Transfer embryos to WASH1; rinse for ~30–60 seconds.
k. Transfer embryos to WASH2; rinse for ~30–60 seconds.
l. Check and record embryo development. Transfer embryos to a CO_2-equilibrated medium embryo culture dish and replace the dish into the CO_2 incubator.

Blastocyst

The cryopreservation of preimplantation human embryos at the blastocyst-stage has become an important part of many ART programs. The following protocol is one of many based on pioneering work by Menezo utilizing glycerol and sucrose as permeating and non-permeating cryoprotectants, respectively (53,54). This protocol utilizes cryo-straws as the cryo-container.

Freezing:

1. Materials (*expendables*): Conical tubes, organ culture dish, 5- and 10-mL disposable pipettes, pulled pipettes—inner diameter just larger then the blastocysts being cryopreserved, 0.2 μm filter, cryo-straws, cryo-canes, cryo-goblets, and cryo-sleeves.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, heat-sealer,

programmable biological freezer, liquid nitrogen storage canister with lock and alarm, safety goggles, and cryo-gloves.

3. Cryo-solutions:

   Freeze medium #1 = HEPES-buffered medium + 12–15% (w/v) protein source (WASH)

   Freeze medium #2 = freeze medium #1 + 5% (v/v) glycerol (5GLYC)

   Freeze medium #3 = freeze medium #1 + 9% (v/v) glycerol + 0.2 M sucrose (9GLYC + SUCR)

4. Procedure
   a. Prepare freeze media inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters prior to use. Filter all freeze media with a 0.2 μM syringe filter.
   b. Label four organ culture dishes with the patient identifiers and one each with WASH1, WASH2, 5GLYC, 9GLYC + SUCR. After rinsing dishes, add 1 mL of the appropriate medium to each dish and keep at room temperature.
   c. Select blastocysts that meet your specific freezing criteria to freeze.
   d. Transfer blastocysts to freeze into WASH1.

      - When transferring blastocysts to a dish, always aspirate a small volume of the medium into which you are moving the blastocysts prior to picking them up.
      - Move the blastocyst(s) in as small of a volume of medium as possible.
      - Place the blastocyst(s) in at least three different areas of the dish to fully rinse them of the last medium.

   e. Transfer blastocyst(s) into WASH2 dish.
   f. Transfer blastocyst(s) into 5GLYC for 10 minutes.
   g. Transfer blastocyst(s) into 9GLYC + SUCR for 10 minutes. During this 10 min period:

      - Label the straw(s), canes(s), goblet(s), and or sleeve(s) with the patient identifiers.
      - Rinse the straw(s) with 9GLYC + SUCR and expel (do not let medium touch pre-sealed end).
      - Load the straw(s) with 9GLYC + SUCR and blastocysts in the following order:

        a. 1-inch 9GLYC + SUCR
        b. 1-inch air space
        c. 1/2-inch 9GLYC + SUCR
        d. 1-inch 9GLYC + SUCR containing blastocysts

e. 1/4-inch air space
f. Fill the remainder of the straw with 9GLYC + SUCR. Make sure the medium completely wets the presealed end of the straw to ensure a proper seal.

- Seal the straw(s)
- Place straw(s) into programmable biological freeze.

h. Once straw(s) are loaded into the programmable freezer, begin the program. The program listed below is just one of many that has been successfully used to cryopreserve blastocyst(s).

Rate (°C/min)	Target temperature (°C)	Hold time (min)
0.0	16.0	0.0
2.0	−7.0	10.0
0.3	−30.0	5.0

i. When the temperature changes to −7°C, hold for five minutes, then seed straw(s) by briefly touching a LN_2-soaked cotton-tipped applicator at the meniscus just below the first airspace.
j. Wait two minutes; inspect the seeded straw(s) to confirm ice crystal formation/growth.
k. When the programmable biological freezer temperature reaches −30°C, it can initiate free-fall to approximately −100°C. Subsequently samples can be removed and plunged into liquid nitrogen. During transfer, use goggles, cryo-protective gloves, and liquid-nitrogen-cooled forceps. Place straw(s) into liquid-nitrogen-submerged goblet on cane, and rapidly transfer into liquid nitrogen storage canister.

Thawing:

1. Materials (*expendables*): Conical tubes, organ culture dish, 5- and 10-mL disposable pipettes, pulled-pipettes—inner diameter just larger then the blastocysts being cryopreserved, 0.2 μm filter, paper tissues.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, Dewar, timer, scissors, straw plunger, safety goggles, and cryo-gloves.
3. Thaw-solutions:

Thaw medium #1 = HEPES-buffered medium + 12–15% (w/v) protein source (TM1 or WASH)
Thaw medium #2 = TM1 + 0.5 M sucrose (0.5SUCR)
Thaw medium #3 = TM1 + 0.2 M sucrose (0.2SUCR)

4. Procedure
   a. Prepare thaw media inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters with HEPES-buffered medium prior to use. Filter all thaw media with a 0.2 μM syringe filter.
   b. Prepare blastocyst culture dishes and place into CO_2 incubator at least four hours before thaw.
   c. Label three organ culture dishes with the patient identifiers and one each with 0.5SUCR, 0.2SUCR, WASH. After rinsing dishes, add 1.0 mL of the appropriate medium to each dish and keep at room temperature.
   d. Transfer the cryo-straw(s) containing blastocysts to be thawed from the liquid nitrogen storage canister to a filled liquid-nitrogen-portable Dewar. Always wear protective goggles and gloves when handling liquid nitrogen. Check the cryo-straw out of the patient embryo logbook and out of the patient's cryopreservation record. Transport the cryo-straw(s) in liquid nitrogen to the site of thaw.
   e. Remove the straw from the liquid-nitrogen-filled Dewar with forceps. Hold the straw horizontally at the sealed, cotton-plugged end and allow the straw to partially thaw at room temperature for 30 seconds.
   f. Gently wipe the condensation off the straw with a paper tissue.
   g. Submerge the straw into a 30°C water bath for 45 seconds.
   h. Remove the straw from the water bath and gently wipe dry with a paper tissue.
   i. Expel the embryos from the straw.

      - Hold the straw vertically over the organ culture dish containing 0.5SUCR with the sealed, cotton-plug end up.
      - Cut the sealed end of the straw, opposite to the cotton-plug end.
      - Cut the cotton-plug end in the middle of the seal.
      - Use the straw plunger to expel the contents of the straw into the organ culture dish containing 0.5SUCR.
      - Move blastocysts to three different areas of the dish and hold for 10 minutes.

j. Transfer blastocysts into 0.2SUCR.

- Use a pulled pipette with diameter just larger than the blastocysts
- Before loading blastocysts into pipette, partially fill the pipette with 0.2SUCR.
- Pick up the blastocysts with the least amount of 0.5SUCR.
- place the blastocysts into 0.2SUCR, pick them up and move (release) them into three separate areas within the 0.2SUCR.

k. Leave the blastocysts in 0.2SUCR for 10 minutes.
l. Move blastocysts into WASH, in a similar manner as mentioned above, and leave for five minutes.
m. Check and record embryo development. Transfer embryos to a CO_2-equilibrated medium embryo culture dish and replace the dish into the CO_2 incubator.

Vitrification

It is important to recognize that while a recent surge in interest of vitrification has been experienced in the field of human ART, this is not a new technology. Pioneering work by the likes of Polge et al. (8), Rall and Fahy (29), and Mazur et al. (55) have provided the foundation for current research and application of vitrification for human oocytes and embryos. In addition, the importance of extensive experience in vitrification of domestic animal oocytes and embryos (56–58) is immeasurable.

Oocytes

Numerous reports of successful human oocyte vitrification, followed by fertilization, embryo development and transfer, and healthy live births exist (Table 2). The following is a human oocyte vitrification protocol that has been successfully used for mouse, bovine, and human oocyte cryopreservation.

Vitrifying:

1. Materials (*expendables*): Conical tubes, tissue culture dish, organ culture dish, 5- and 10-mL disposable pipettes, pulled-pipettes—inner diameter just larger than the oocytes being cryopreserved, 0.2 μm filter, 20 μl pipette tips, cryo-goblet, cryo-cane, cryo-sleeve, 1-cm^3 syringe, “pulled-straw” with an inner diameter of approximately 200 μm, syringe–“pulled-straw” connector.

Table 2 Live Births from Vitrified Human Oocytes (Empty Cells Indicate Data Not Available)

Authors	Year	Method		Survival (%)	Fertilization (%)	Infants born (#)
		Cryoprotectants	Device			
Kuleshova et al. (59)	1999	EG, sucrose	OPS	65	45	1
Yoon et al. (60)	2003	EG, sucrose	EM grid	69	72	7
Katayama et al. (61)	2003	DMSO, EG, sucrose	Cryo-top	94	91	1
Kuwayama et al. (31)	2005	DMSO, EG, sucrose	Cryo-top	91	90	7
Total documented live births						16

Abbreviations: DMSO, dimethylsulfoxide, EG, ethylene glycol; OPS, open-pulted straw; EM, electron microscopy.

2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, heat-sealer, forceps, liquid nitrogen Dewar, liquid nitrogen storage canister with lock and alarm, safety goggles, and cryo-gloves.
3. Cryo-solutions:
   Wash solution = HEPES-buffered medium + 12% (w/v) protein source (WASH)
   Equilibration solution (ES) = HEPES-buffered medium + 7.5% (v/v) ethylene glycol + 7.5% DMSO + 12% (w/v) protein source
   Vitrification solution (VS) = HEPES-buffered medium + 15% (v/v) ethylene glycol + 15% DMSO + 0.5 M sucrose + 12% (w/v) protein source
4. Procedure
   a. Prepare vitrification media inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters prior to use. Filter all cryo-media with a 0.2 μM syringe filter. Prepare oocyte culture dish at least four hours before use and allow to equilibrate in CO_2 incubator.
   b. Warm WASH, ES, and VS to room temperature. Make certain the solutions are mixed and homogeneous.
   c. For each straw of oocytes being vitrified, prepare a culture dish with patient identifiers and written indication of 20-μL drop placements. In addition, label the "pulled-straw" with proper patient identifiers.
   d. Immediately before beginning the procedure, dispense 20 μL drops of WASH (one drop), ES (three drops), and VS (four drops) onto the culture dish (Fig. 2). Alternatively, the VS drops can be dispensed when oocytes are in the ES3 drop.
   e. With a pulled-pipette and the least amount of medium carry-over, transfer denuded metaphase II (MII) oocytes from culture medium into WASH drop for one minute.
   f. Using the pulled-pipette tip, merge the ES1 drop with WASH drop in a dragging motion from ES1 to WASH. There is no need to move oocytes. Set timer for two minutes.
   g. When timer sounds, merge ES2 drop with the WASH/ ES1 drop in a similar manner, dragging from ES2 toward WASH. Set timer for two minutes.
   h. When timer sounds, transfer oocytes using a pulled-pipette from merged drops to ES3 drop. Leave in ES3 for three minutes. During this three minute period, prepare the pulled straw for loading, sealing, and plunging.

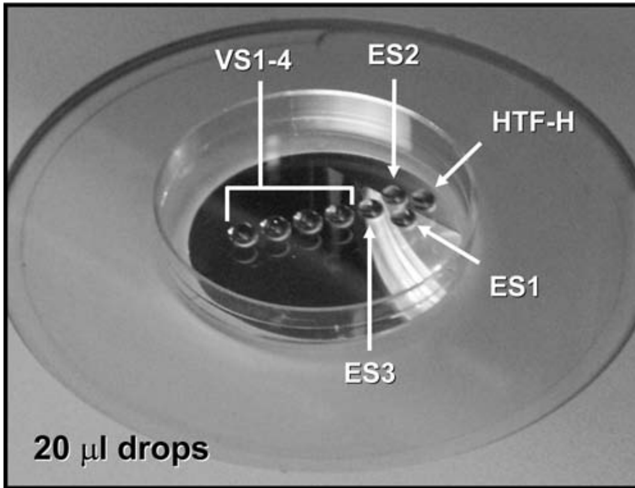

Figure 2 Photograph representation of solution drop orientation used for oocyte vitrification. Drops are 20 μL each and at room temperature. *Abbreviations*: HTF-H, human tubal fluid—HEPES medium; ES, equilibration solution; VS, vitrification solution.

i. Using a pulled-pipette, transfer oocyte through the VS drops. This should be done with microscope visualization and manual counting.
   - Move oocytes into the VS1 drop for five seconds.
   - Move oocytes into the VS2 drop for five seconds.
   - Move oocytes into the VS3 drop for 10 seconds.
   - Move oocytes into the VS4 drop.

j. The goal at this point is to load the pulled-straw, seal the straw, and plunge the straw in liquid nitrogen within a 90-second interval.
   - To load the pulled straw, aspirate VS4 into straw to the first line (closest to the fine-pulled tip), aspirate VS4 and oocytes so that the fluid meniscus reaches the second line, aspirate additional VS4 so that the fluid meniscus reaches the third line (Fig. 3).
   - Heat-seal the narrow end of the straw just below the first mark, and then above the fourth mark.
   - Hold the sealed straw with forceps and immerse, while swirling, directly into liquid nitrogen.

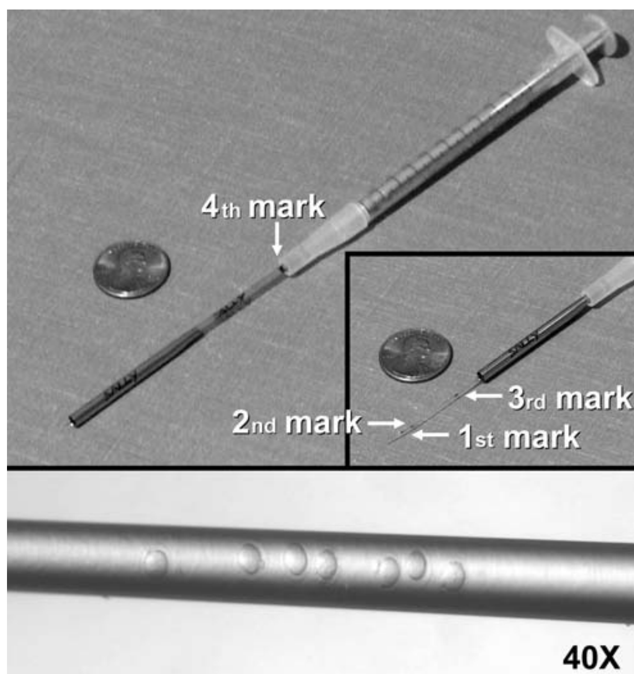

Figure 3 Photographs and micrograph demonstrating the size and line marks on the closed-pulled straw used to vitrify oocytes, zygotes, cleavage-stage embryos, and blastocysts. The bottom panel demonstrates the size of the fine-pulled end of the straw containing mouse MII oocytes.

Warming:

1. Materials (*expendables*): Conical tubes, tissue culture dish, organ culture dish, 5- and 10-mL disposable pipettes, pulled-pipettes—inner diameter just larger than the oocytes being cryopreserved, 0.2 μm filter, 20 μL pipette tips, 1 cm^3 syringe, syringe/pulled-straw connector, paper tissue, or sterile gauze.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, forceps, thermometer, water bath, scissors, liquid nitrogen Dewar, safety goggles, and cryo-gloves.
3. Warming-solutions:
   Initial warming solution = HEPES-buffered medium + 1.0 M sucrose + 12% (w/v) protein source (IWS)
   Dilution solution = HEPES-buffered medium + 0.5 M sucrose + 12% (w/v) protein source (DS)

Wash solution = HEPES-buffered medium + 12% (w/v) protein source (WASH) (VS)

4. Procedure
   a. Prepare warming solutions inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters prior to use. Filter all warming media with a 0.2 μM syringe filter. Prepare oocyte culture dishes at least four hours before use and allow to equilibrate in the CO_2 incubator.
   b. Warm IWS, DS, and WASH to room temperature. Make certain the solutions are mixed and homogeneous.
   c. For each straw of oocytes being warmed, prepare a culture dish with patient identifiers and written indication of 20 μL drop placements.
   d. Immediately before beginning the procedure, dispense 20 μL drops of IWS (one drop), DS (two drops) onto the culture dish.
   e. Before beginning the warming process, ensure that all equipment and expendables are accounted for and functioning. Select the straw of oocytes to be warmed and rapidly transfer (in goblet containing liquid nitrogen) from the liquid nitrogen storage tank to liquid-nitrogen-filled Dewar. Place the Dewar close to the 37°C water bath.
   f. Begin the warming process.
      - With forceps, remove the close-pulled straw with oocytes from the liquid nitrogen and immediately submerge completely into the 37°C water bath while swirling the straw for three to five seconds.
      - Remove from the water bath and wipe dry with paper tissue or sterile gauze.
      - Using scissors, cut the straw at the end near the fourth mark at the large end of the straw.
      - Attach to pipetting device.
      - Position the straw tip over the culture dish and using scissors, cut the straw between the first and second marks but closest to the first mark.
      - Dispense the pulled-straw contents as a small drop onto the culture dish; avoid bubbles.
      - Rinse the pulled-straw with contents from the IWS drop by aspiration up to the third mark. Dispense this rinse IWS as a small drop next to the initial pulled-straw contents and merge the two drops together with a dragging motion of the pulled-straw. Set the timer for one minute.

g. When timer sounds, transfer oocytes using a pulled-pipette to the bottom of the 20 μL IWS drop. Set the timer for one minute.
h. When timer sounds, transfer oocytes using a pulled-pipette to the bottom of the DS1 drop for two minutes, then transfer to DS2 drop for two minutes. During the DS2 exposure, dispense three 20 μL drops of WS.
i. Using a pulled-pipette, transfer the oocyte through the WASH (1, 2, and 3) drops, with a two-minute exposure in each drop. In the final WASH, drop check and record survival/development.
j. Transfer oocytes into CO_2-equilibrated culture media, place into CO_2 incubator, and perform ICSI four hours after warming.

Zygotes, Cleavage-Stage Embryos, and Blastocysts

The ability to vitrify human pronuclear-stage zygotes, cleavage-stage embryos, and blastocysts has been demonstrated by many laboratories. The protocol listed below is just one of many that have been demonstrated to produce quite acceptable results for vitrification of all stages of preimplantation human embryos (62).

Vitrifying:

1. Materials (*expendables*): Conical tubes, tissue culture dish, organ culture dish, 5- and 10-mL disposable pipettes, pulled-pipettes—inner diameter just larger than the cells being cryopreserved, 0.2 μm filter, 20 μL pipette tips, cryo-goblet, cryo-cane, cryo-sleeve, 1 cm^3 syringe, "pulled-straw" with an inner diameter of approximately 200–230 μm, syringe–"pulled-straw" connector.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, heat-sealer, forceps, liquid nitrogen Dewar, liquid nitrogen storage canister with lock and alarm, safety goggles, and cryo-gloves.
3. Cryo-solutions:
   Wash solution = HEPES-buffered medium + 12% (w/v) protein source (WASH)
   Equilibration solution = HEPES-buffered medium + 7.5% (v/v) ethylene glycol + 7.5% DMSO + 12% (w/v) protein source (ES)
   Vitrification solution = HEPES-buffered medium + 15% (v/v) ethylene glycol + 15% DMSO + 0.5 M sucrose + 12% (w/v) protein source (VS)
4. Procedure
   a. Prepare vitrification media inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters prior

to use. Filter all cryo-media with a 0.2 μM syringe filter. Prepare embryo culture dish at least four hours before use and allow to equilibrate in the CO_2 incubator.

b. Warm WASH, ES, and VS to room temperature. Make certain the solutions are mixed and homogeneous.
c. For each straw of zygotes/embryos being vitrified, prepare a culture dish with patient identifiers and written indication of 20 μL drop placements. In addition, label the "pulled-straw" with proper patient identifiers.
d. Immediately before beginning the procedure, dispense 20 μL drops of WASH (one drop), ES (one drop) onto the culture dish.
e. Select zygotes/embryos for vitrification that meet your cryopreservation criteria. Using a pulled-pipette and the least amount of media carry-over, transfer cells (maximum two at a time) from culture media into WASH drop for one minute.
f. Using the pulled-pipette tip, transfer zygotes/embryos into the top of the ES drop. The cells will begin to shrink and sink to the bottom of the drop. Zygotes/embryos with gradually return to their original size. Leave the zygotes/embryos in this ES drop for five to 15 minutes or until they re-expand to original size. During this equilibration step, dispense four 20 μL drops of VS (VS1–VS4).
g. Transfer zygotes/embryos from ES to the VS1 drop. Microscopically observe zygotes/embryos and count to five seconds.
h. Transfer zygotes/embryos to VS2 for five seconds.
i. Transfer zygotes/embryos to VS3 for 10 seconds.
j. Transfer zygotes/embryos to VS4. The goal at this point is to load the pulled-straw, seal the straw, and plunge the straw in liquid nitrogen within a 90 seconds interval.

   - To load the pulled straw, aspirate VS4 into straw to the first line (closest to the fine-pulled tip), aspirate VS4 and zygotes/embryos with fluid meniscus reaching the second line, aspirate additional VS4 with fluid meniscus reaching the third line.
   - Heat-seal the narrow end of the straw just below the first mark, and then above the fourth mark.
   - Hold the sealed straw with forceps and immerse, while swirling, directly into liquid nitrogen.

Warming:

1. Materials (*expendables*): Conical tubes, tissue culture dish, organ culture dish, 5- and 10-mL disposable pipettes, puller-pipettes—inner diameter just larger than the cells being cryo-preserved, 0.2 μM filter, 20 μL pipette tips, 1-cm^3 syringe, syringe/pulled-straw connector, paper tissue or sterile gauze.
2. Equipment: Pipetting devices, laminar-flow hood, dissecting microscope with lighted base, labeling device, timer, forceps, thermometer, water bath, scissors, liquid nitrogen Dewar, safety goggles, and cryo-gloves.
3. Warming-solutions:
   Initial warming solution = HEPES-buffered medium + 1.0 M sucrose + 12% (w/v) protein source (IWS)
   Dilution solution + HEPES-buffered medium + 0.5 M sucrose + 12% (w/v) protein source (DS)
   Wash solution = HEPES-buffered medium + 12% (w/v) protein source (WASH)
4. Procedure
   a. Prepare warming solutions inside the hood at least one day prior to use. Rinse all tubes, syringes, and filters prior to use. Filter all cryo-media with a 0.2 μM syringe filter. Prepare zygote/embryo culture dishes at least four hours before use and allow to equilibrate in the CO_2 incubator.
   b. Warm IWS, DS, and WASH to room temperature. Make certain the solutions are mixed and homogeneous.
   c. For each straw of zygotes/embryos being warmed, prepare a culture dish with patient identifiers and written indication of 20 μL drop placements.
   d. Immediately before beginning the procedure, dispense 20 μL drops of IWS (one drop) DS (two drops) on to the culture dish.
   e. Before beginning the warming process, ensure that all equipment and expendables are accounted for and functioning. Select the straw of zygotes/embryos to be warmed and rapidly transfer (in goblet containing liquid nitrogen) from the liquid nitrogen storage tank to liquid-nitrogen-filled Dewar. Place the Dewar close to the 37°C water bath.
   f. Begin the warming process.
      - With forceps, remove the close-pulled straw with zygotes/embryos from the liquid nitrogen and immediately and completely submerge into the 37°C

water bath while swirling the straw for three to five seconds.
- Remove from the water bath and wipe dry with a paper tissue or sterile gauze.
- Using scissors, cut the straw at the end near the fourth mark at the large-end of the straw.
- Attach to pipetting device.
- Position the straw tip over the culture dish and using scissors, cut the straw between the first and second marks but closest to the first mark.
- Dispense the pulled-straw contents as a small drop onto the culture dish; avoid bubbles.
- Rinse the pulled-straw with contents from the IWS drop by aspiration up to the third mark. Dispense this rinse IWS as a small drop next to the initial pulled-straw contents and merge the two drops together with a dragging motion of the pulled-straw. Set the timer for one minute.

g. When timer sounds, transfer zygotes/embryos using a pulled-pipette to the bottom of the 20 μL IWS drop. Set the timer for one minute.
h. When timer sounds, transfer zygotes/embryos using a pulled-pipette to the bottom of DS1 drop for two minutes, then transfer to DS2 drop for two minutes. During the DS2 exposure, dispense three 20 μL drops of WS.
i. Using a pulled-pipette, transfer zygotes/embryos through the WASH (1, 2, and 3) drops, with two minutes exposure in each drop. In the final WASH drop, check and record survival/development.
j. Transfer zygotes/embryos into culture media, place into incubator.

TROUBLESHOOTING AND A SUCCESSFUL CRYOPRESERVATION PROGRAM

As a preface, it is fundamental to recognize that clinical human oocyte/embryo cryopreservation programs are inherently difficult to troubleshoot and improve because of material used (usually not the best embryos) and passage of time between cryopreservation and oocyte/embryo utilization. All would likely agree that a successful embryo cryopreservation program is pivotal in maximizing the cumulative pregnancy rate from a single oocyte retrieval. However, it is also essential that one be realistic in expectations of a cryopreservation program. Even with embryos derived from donor

oocytes, the pregnancy rate of recipients receiving thawed embryos has been reported to be approximately 60% of that of recipients receiving fresh embryos (63).

In general terms, a key factor that dictates success of oocyte/embryo cryopreservation is the condition and quality of the starting material. This is likely true independent of the method of cryopreservation. Having ART-program-specific cryopreservation criteria are important. They can be determined by reviewing past success of pregnancies and live births of cryopreserved embryos of different grades. It is inherently obvious that "poor-quality in" and "poor-quality out" applies to embryo and likely oocyte cryopreservation. Rate of cell division and degree of fragmentation are important considerations in determining expectations of any cryopreservation protocol. Lastly, there are likely numerous intracellular functions and cell-cycle-dependent events that are susceptible to cryo-damage, influence cryopreservation success, and yet are currently underappreciated (26).

Attempting to troubleshoot or improve success of cryo-protocols requires attention to specific steps within a cryo-protocol. This holds true for both slow-rate freezing/thawing and vitrification/warming. There is no doubt the "devil is in the details." The following are just a few of many possible practical points to consider when attempting to optimize cryopreservation protocols. In all cases, it is prudent to systematically implement single alterations and, whenever possible, experimentally test such alterations in an animal model system before implementing them into use within the clinical laboratory.

Proteins can act as osmotic regulators within cryo-solutions. Because "osmotic shock" can be a critical determinant of cell survival during freezing/thawing or vitrification/warming, the concentration of protein in cryo-solutions should be considered. Many successful cryopreservation protocols will utilize protein addition at 12–15 mg/mL (64).

Inclusion of a higher concentration of sucrose or similar non-permeating cryoprotectant in the initial thaw/warming solution compared to the final concentration of non-permeating cryoprotectant in the freezing/vitrifying solution is believed to be important to enhanced cryo-survival. Such elevations in non-permeating cryoprotectants in the thawing/warming solutions are seen in most of the protocols above, with the exception of the oocyte slow-rate freezing protocol which is based on work from the Italian group of Porcu et al. (41,52). These investigators demonstrated that cryo-survival of oocytes slow-rate cryopreserved and thawed in 0.3 M sucrose was significantly better than 0.2 or 0.1 M sucrose. However, these investigators did not test the comparison of lower sucrose in freeze medium in relation to thaw medium. Boldt et al. (49) reported a commendable cryo-survival rate of 74% using human MII oocytes and slow-rate freezing in sodium-depleted cryopreservation medium where sucrose concentrations were elevated in thaw solutions compared to freeze solutions. The replacement of sodium

chloride with choline chloride was demonstrated previously to be beneficial for mouse oocyte slow-rate cryopreservation (65–67) and likely holds great promise in improving cryo-survival of human oocytes.

A critical aspect of sequential cryoprotectant exposure is attention to the means of cell movement, solution carry-over, solution dissociation, and fluid microenvironments. As one can note in the above protocols, movement from a solution of one concentration of cryoprotectant to another should use a pulled-pipette just larger than the cells being transferred. This reduces cryoprotectant carry-over. Before transfer, one should also aspirate a small amount of solution in which the cells will be moved prior to taking up the cells. This allows better pipetting control and reduces carry-over of the previous solution into the subsequent solution. Once cells are moved into the next cryo-solution, it is recommended that they be moved through a few different areas of the solution. This minimizes formation of a fluid microenvironment of the previous solution surrounding the cells. The above-mentioned cautions can help ensure exposure of cells to the desired concentration of cryoprotectant for the proper amount of time.

Slow-rate freezing and seeding is often an area of concern and discussion. When using vials, seeding at the cryo-solution meniscus is recommended. If straws are oriented in a vertical fashion and one ensures that cells are not near the top meniscus, one can seed at the top meniscus of this solution column. When seeding, ensure good ice nucleation. This can be done with liquid-nitrogen-supercooled forceps or cotton swabs of a wooden stick applicator. The device should be supercooled between each seeding. Lastly, at approximately two minutes after seeding, one should rapidly visualize the seeded area to ensure that ice nucleation is still present and growing.

Finally, with regards to vitrification of oocytes and/or embryos, it is essential that the technologist practice and demonstrate a high degree of proficiency in cell movement through solutions of very different viscosities and buoyancies. This should be done with cells obtained from experimental model systems (mouse, hamster, bovine, 3PN zygotes, etc.). For successful vitrification, it is essential that one be extremely well prepared and has all solutions, expendables, and equipment at hand before starting the procedure. Once the vitrification or warming has been started, there is very little time for mistakes or misplacement of a procedural component.

FUTURE OF GAMETE/EMBRYO CRYOBIOLOGY

Although cryobiology has theoretical and experimental roots anchored in the nineteenth and early twentieth centuries, the major experimental and applicable advances of gamete and embryo cryobiology have been made in the last 60 years. The same might be said of numerous other areas of developmental biology, biochemistry, cell biology, genetics, and molecular biology. Since the 1950s, there have been astonishing shifts in scientific

paradigms in all these areas of science. Advancements in cryopreservation of mammalian gametes and embryos have had an enormous impact on clinical ARTs in the last three decades. When one thinks back in history, it is remarkable to consider that these advancements are based, at least philosophically, on techniques and protocols pioneered by the likes of Polge, Audrey Smith, and James Lovelock in the 1950s. So the question arises: what might one expect in the future? It is quite likely that vitrification of gametes and embryos will gain a more widespread acceptance. Again, it is essential to appreciate that the practical advantages of vitrification have been appreciated for numerous decades. If one considers the views expressed by the prominent cryobiologist Luyet in his seminal work of *Life and Death at a Low Temperature* (68), it is easy to capture his contemporary view of vitrification:

> Good vitrification is not injur[i]ous, there being no molecular disturbance, while an incomplete vitrification or devitrification and, a fortiori, crystallization, are injur[i]ous to the extent that they disrupt the living structure (68).

The advantages of vitrification are still acknowledged today, as expressed by Taylor and colleagues:

> A vitrified liquid is essentially a liquid in molecular stasis. Vitrification does not have any of the biologically damaging effects associated with freezing because no degradation occurs over time in living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biological systems (69).

When one cryopreserves millions of cells, such as transformed cell lines, sperm, or even tissues, the significant lose of a percentage of cells may not be viewed as critical for subsequent usage. This is not the case when one considers cryopreservation of a small finite set of cells or group of cells, such as oocytes and embryos. These cells require the most efficient means of cryopreservation and retrieval of viability. While slow-rate freezing has been successful, one has to ask whether vitrification will have added benefit.

Advancements in the area of "freeze-drying" platelets (70) and sperm (71–74) are very exciting and beg the question of whether such techniques might have future application in long-term storage of oocytes and embryos. The potential to provide such storage in a freeze-dried state has numerous advantages, one being the actual means of long-term storage without liquid nitrogen. Whether such advances will be made for oocytes and embryos remains to be seen.

The fields of infertility treatment and ARTs are indebted to the numerous cryobiologists, whose basic and translational research has contributed significantly to the current success of gamete and embryo cryopreservation.

With this said, there is always room for improvement. Knowledge continues to accumulate regarding intricacies of cell biology, regulation of gene expression, factors influencing epigenetic modifications, and how all these cellular functions culminate into normal physiology. The last 50 year have been exciting times in cryobiology; one could venture an opinion that the next fifty years may provide currently inconceivable advancements, that once applied, will lead to betterment of health and increased happiness.

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REFERENCES

1. Leibo SP. The early history of gamete cryobiology. In: Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press, 2004:347–370.
2. Smith AU. Biological Effects of Freezing and Supercooling. Baltimore: Williams and Wilkins, 1961.
3. Ohl DA, Park J, Cohen J, Goodman K, Menge AC. Procreation after death or mental incompetence: medical advance or technology gone awry? Fertil Steril 1996; 66:889–895.
4. Finnerty JJ, Thomas TS, Boyle RJ, Howards SS, Karns LB. Gamete retrieval in terminal conditions. Am J Obstet Gynecol 2001; 185:300–307.
5. Tash JA, Applegarth LD, Kerr SM, Fins JJ, Rosenwaks Z, Schlegel PN. Postmortem sperm retrieval: the effect of instituting guidelines. J Urol 2003; 170:1922–1925.
6. Hammond J. The effect of temperature on the survival in vitro of rabbit spermatozoa obtained from the vagina. J Exp Biol 1930; 7:175–191.
7. Walton A. The effect of temperature on the survival in vitro of rabbit spermatozoa obtained from the vas deferens. J Exp Biol 1930; 7:201–219.
8. Polge E, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949; 164:666.
9. Bunge RG, Sherman JK. Fertilization capacity of frozen human spermatozoa. Nature 1953; 172:767–768.
10. Sherman JK, Bunge RG. Observations on preservation of human spermatozoa of low temperatures. Proc Soc Exp Biol Med 1953; 82:686.
11. Chang MC. The effect of low temperature on fertilized rabbit ova in vitro, and the normal development of ova kept at low temperature for several days. J Gen Physiol 1948; 31:385–410.

12. Chang MC. Fertilizability of rabbit ova and the effect of temperature in vitro on their subsequent fertilization and activation in vivo. J Exp Zoo 1952; 121: 351–381.
13. Lin TP, Sherman JK, Willet EL. Survival of unfertilized mouse eggs in media containing glycerol and glycine. J Exp Zoo 1957; 134:275–297.
14. Sherman JK, Lin TP. Effect of glycerol and low temperature on survival of unfertilized mouse eggs. Nature 1958; 181:785–786.
15. Sherman JK, Lin TP. Survival of unfertilized mouse eggs during freezing and thawing. Proc Soc Exp Biol Med 1958; 98:902–905.
16. Sherman JK, Lin TP. Temperature shock and cold-storage of unfertilized mouse eggs. Fertil Steril 1959; 10:384–396.
17. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J Gen Physiol 1963; 47:347–369.
18. Mazur P. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. Cell Biophys 1990; 17:53–92.
19. Mazur P, Leibo SP, Chu EHY. A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells. Exp Cell Res 1972; 71:345–355.
20. Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to −196 and −269°C. Science 1972; 178:411–414.
21. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. Nature 1983; 305:707–709.
22. Zeilmaker GH, Alberda AT, van Gent I, Rijkmans CM, Drogendijk AC. Two pregnancies following transfer of intact frozen-thawed embryos. Fertil Steril 1984; 42:293–296.
23. Testart J, Lassalle B, Belaisch-Allart J, et al. High pregnancy rate after early human embryo freezing. Fertil Steril 1986; 46:268–272.
24. Pegg DE. Mechanisms of freezing damage. Symp Soc Exp Biol 1987; 41:363–378.
25. Bernard A, Fuller BJ. Cryopreservation of human oocytes: a review of current problems and perspectives. Hum Reprod Update 1996; 2:193–207.
26. Smith GD, Silva E Silva CA. Symposium: cryopreservation and assisted human conception developmental consequences of cryopreservation of mammalian oocytes and embryos. Reprod BioMed Online 2004; 9:171–178.
27. Meryman HT. Cryoprotective agents. Cryobiology 1971; 8:173–183.
28. Friedler S, Giudice LC, Lamb EJ. Cryopreservation of embryos and ova. Fertil Steril 1988; 49:743–764.
29. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature 1985; 313:573–575.
30. Rall WF. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology 1987; 24:387–402.
31. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod BioMed Online 2005; 11:300–308.
32. Isachenko V, Montag M, Isachenko E, et al. Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. Hum Reprod 2005; 20:492–496.
33. El-Danasouri I, Selman H. Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. Fertil Steril 2001; 76:400–402.

34. Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryo-loop container-less technique. Fertil Steril 1999; 72:1073–1078.
35. Liebermann J, Tucker MJ, Graham JR, Han T, Davis A, Levy MJ. Blastocyst development after vitrification of multipronuclear zygotes using flexipet denuding pipette. Reprod BioMed Online 2002a; 4:146–150.
36. Mukaida T, Takahashi K, Kasai M. Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. Reprod BioMed Online 2003; 6:221–225.
37. Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. Fertil Steril 2002; 78:449–454.
38. Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential importance of vitrification in reproductive medicine. Biol Reprod 2002; 67:1671–1680.
39. Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986; 327: 884–886.
40. Van Uem JFHM, Siebzehnrubl ER, Schuh B, Koch R, Trotnow S, Lang N. Birth after cryopreservation of unfertilised oocytes. Lancet 1987; 329:752–753.
41. Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. Fertil Steril 1997; 68:724–726.
42. Porcu E, Fabbri R, Seracchioli R, et al. Birth of six healthy children after intracytoplasmic sperm injection of cryopreserved human oocytes. Hum Reprod 1998; 13:124.
43. Tucker MJ, Morton PC, Wright G, Sweitzer CL, Massey JB. Clinical application of human egg cryopreservation. Hum Reprod 1998; 13:3156–3159.
44. Polak de Fried E, Notrica J, Rubinstein M, Marazzi A, Gomez Gonzalez M. Pregnancy after human donor oocyte cryopreservation and thawing in association with intracytoplasmic sperm injection in a patient with ovarian failure. Fertil Steril 1998; 69:555–557.
45. Porcu E, Fabbri R, Damiano G, et al. Clinical experience and applications of oocyte cryopreservation. Mol Cell Endocrinol 2000; 169:33–37.
46. Winslow KL, Yang D, Blohm PL, Brown SE, Jossim P, Nguyen K. Oocyte cryopreservation a three year follow up of sixteen births. Fertil Steril 2001; 76: S120–S121.
47. Quintans CJ, Donaldson MJ, Bertolino MV, Pasqualini RS. Birth of two babies using oocytes that were cryopreserved in a choline-based freezing medium. Hum Reprod 2002; 17:3149–3152.
48. Yang D, Winslow KL, Blohm PL, Brown SE, Nguyen K, Brubaker C. Oocyte donation using cryopreserved donor oocytes. Fertil Steril 2002; 78:S14–S15.
49. Boldt J, Cline D, McLaughlin D. Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. Hum Reprod 2003; 18:1250–1255.
50. Fosas N, Marina F, Torres PJ, et al. The births of five Spanish babies from cryopreserved donated oocytes. Hum Reprod 2003; 18:1417–1421.
51. Borini A, Bonu MA, Coticchio G, Bianchi V, Cattoli M, Flamigni C. Pregnancies and births after oocyte cryopreservation. Fertil Steril 2004; 82:601–605.

52. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 2001; 16:411–416.
53. Menezo Y, Nicollet B, Herbaut N, Andre D. Freezing cocultured human blastocyst. Fertil Steril 1992; 58:977–980.
54. Menezo Y, Nicollet B, Dumont M, Hazout A, Janny L. Factors affecting human blastocyst formation in vitro and freezing at the blastocyst stage. Act Eur Fertil 1993; 24:207–213.
55. Mazur P, Cole KW, Hall WH, Schreuders PD, Mahowald AP. Cryobiological preservation of Drosophila embryos. Science 1992; 258:1932–1935.
56. Lopez-Gatius F, Camon-Urgel J. Pregnancies and live offspring following transfer of one-step vitrified bovine embryos. Zuchthygiene 1989; 24:255–258.
57. Tachikawa S, Otoi T, Kondo S, Machida T, Kasai M. Successful vitrification of bovine blastocysts, derived by in vitro maturation and fertilization. Mol Reprod Dev 1993; 34:266–271.
58. Agca Y, Monson RL, Northey DL, Mazni OA, Schaefer DM, Rutledge JJ. Transfer of fresh and cryopreserved IVP bovine embryos: normal calving, birth weight and gestation lengths. Theriogenology 1998; 50:147–162.
59. Kuleshova L, Gianaroli L, Magli M, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes. Hum Reprod 1999; 14: 3077–3079.
60. Yoon TK, Kim TJ, Park SE, et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization–embryo transfer program. Fertil Steril 2003; 79:1323–1326.
61. Katayama KP, Stehlik J, Kuwayama M, Kato O, Stehlik E. High survival rate of vitrified human oocytes results in clinical pregnancy. Fertil Steril 2003; 80:223–224.
62. Kuwayama M, Vajta G, Leda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod BioMed Online 2005b; 11:608–614.
63. Check JH, Choe JK, Nazari A, Fox F, Swenson K. Fresh embryo transfer is more effective than frozen for donor oocyte recipients but not for donors. Hum Reprod 2001; 16:1403–1408.
64. Veeck LL, Bodine R, Clarke RN, et al. High pregnancy rates can be achieved after freezing and thawing human blastocysts. Fertil Steril 2004; 82:1418–1427.
65. Stachecki JJ, Cohen J, Willadsen SM. Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. Cryobiology 1998; 37:346–354.
66. Stachecki JJ, Cohen J, Willadsen SM. Detrimental effects of sodium during mouse oocytes cryopreservation. Biol Reprod 1998; 59:395–400.
67. Stachecki JJ, Cohen J, Schimmel T, Willadsen SM. Fetal development of mouse oocytes and zygotes cryopreserved in a nonconventional freezing medium. Cryobiology 2002; 44:5–13.
68. Luyet BJ, Gehenio PM. Life and Death at Low Temperatures. Normandy, MO: Biodynamica, 1940:341.

69. Taylor MJ, Song YC, Brockbank KGM. Vitrification in tissue preservation: new developments. In: Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press, 2004:603–644.
70. Wolkers WF, Walker NJ, Tablin F, Crowe JH. Human platelets loaded with trehalose survive freeze-drying. Cryobiology 2001; 42:79–87.
71. Wakayama T, Yanagimachi R. Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nature Biotechnol 1998; 16:639–641.
72. Keskintepe L, Pacholczyk G, Machnicka A, et al. Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa. Biol Reprod 2002; 67:409–415.
73. Ward MA, Kaneko T, Kusakabe H, Biggers JD, Whittingahm DG, Yanagimachi R. Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection. Biol Reprod 2003; 69:2100–2108.
74. Liu JL, Kusakabe H, Chang CC, et al. Freeze-dried sperm fertilization leads to full-term development in rabbits. Biol Reprod 2004; 70:1776–1781.

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Quality Assurance and Quality Control in the In Vitro Fertilization Laboratory

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INTRODUCTION

The main objective of any in vitro fertilization (IVF) unit is to provide good service for their patients. Although this can be interpreted in several ways, it is usually perceived as achieving a live birth (1). One of the most important factors contributing to this outcome is the performance of the IVF laboratory responsible for the creation of embryos from patients' gametes. The viability of the embryos created and transferred into the woman's uterus is a major determinant in establishing a successful pregnancy. As the development and viability of gametes and embryos in vitro is compromised by contaminants and relatively small fluctuations in the environment, such as temperature and pH (2,3), it is imperative that optimal culture conditions for gametes and embryos are attained and maintained in the IVF laboratory to ensure the best care for patients. The purpose of employing quality assurance (QA) and quality control (QC) schemes is to ensure reproducibility in all methods and performance within the IVF laboratory (4), and thus provide consistent, optimal conditions for gamete and embryo culture. Moreover, the diagnostic as well as the therapeutic role of the IVF laboratory can benefit from employing QA and QC programs (5).

WHAT ARE QA AND QC?

QC involves measures and activities undertaken to control the quality of products, methods, equipment, and environment to ensure that the laboratory is functioning correctly, and is carried out to prevent undetected problems leading to a compromise in service to the patient. Internal quality control (IQC) refers to those measures undertaken on site.

QA is a comprehensive program that includes all activities and programs intended to ensure or improve the performance of the laboratory and thus the quality of care to patients. QA includes measures such as record keeping, evaluation and education of laboratory staff, results reporting, treatment auditing, incident reporting, etc., as well as QC methodology.

Both QA and IQC are systems that look within a particular testing site. In comparison, external quality assessment (EQA) looks at differences between different sites testing the same analyte to ensure continuity of patient care between institutions and/or to ensure standards meet those of accredited/regulatory bodies.

WHY EMPLOY QA AND QC SCHEMES IN THE IVF LABORATORY?

Until relatively recently, QA and QC programs were little employed within IVF laboratories despite their obligatory use and proven benefits in other types of clinical facilities. Although this was originally attributed to IVF being a new field of medicine which did not fall under the auspices of any accreditation/regulatory authorities, 25 years later, changing legislation in some countries and/or the need to raise standards to compete with other units is leading to greater numbers of IVF laboratories employing QA and QC schemes. Furthermore, QA and QC programs have not only been shown to benefit the running of an existing IVF laboratory (4,5), but are also invaluable when setting up new facilities (6). The benefits and application of QA and QC schemes will therefore be discussed in relation to both the new and the established IVF unit.

Benefits of Employing QA and QC Schemes When Setting Up a New IVF Laboratory

When setting up a new IVF laboratory, or even making alterations to an existing laboratory, there are usually a large number of new and untested variables which should be validated before use in clinical practice. This may apply to equipment, methodology, environment, consumables, and/or culture media. The use of QA and QC to test these new variables will ensure that optimal standards of practice are attained from the outset in the new IVF laboratory, thus ensuring that patient treatment is not compromised. In a prospective analysis, Cutting et al.(6) demonstrated that

employment of a QC scheme identified problems with equipment before treatment commenced when setting up the Assisted Conception Unit (ACU), Center for Reproductive and Developmental medicine (CRMF), Sheffield Teaching Hospitals Foundation Trust, Jessop Wing, Sheffield, U.K. These problems are likely to have led to a compromise in pregnancy rates if they had gone unchecked. As such, QC can give confidence to the scientific staff and thus the whole IVF team that gamete and embryo development are not compromised by new laboratory facilities and procedures.

The use of EQA where possible is also important to ensure that laboratory practices and personnel in the new IVF laboratory are comparable to acceptable standards of other laboratories or preferably accredited/regulatory bodies. Again this ensures that patient treatment is not compromised at the expense of a learning curve and gives confidence to the IVF team from the outset.

Benefits of Employing QA and QC Schemes in an Established IVF Laboratory

There are several advantages of employing QA and QC schemes in the established IVF laboratory. These include ensuring optimal laboratory performance is consistent and maintained. Their use also allows rapid and effective troubleshooting should there be a problem such as a decrease in fertilization rate. As such, QC especially allows the staff to determine whether the cause lies within the laboratory, and if so, where the problem resides (4). Identifying problems through QC should therefore lead to an improvement in work processes and protocols. More importantly, a QC scheme should detect problems before they become significant and impact on the care of patients.

In addition to the above, QA and QC are also useful when incorporating new technologies and/or methodologies into the established IVF laboratory and thus for improving laboratory performance. This is particularly applicable to the field of assisted reproductive technology as new developments are frequently emerging. Validation and monitoring of new methods and techniques is important as the introduction of new procedures can compromise the quality of service to patients. The effectiveness of new techniques and protocols can be readily assessed with QA and QC measures giving confidence to the team upon implementation of the new procedures. In turn, moving forward confidently with new developments educates and thus motivates the team as well as improving patient care.

The use of EQA in the existing laboratory is important for evaluating personnel performance as part of their career development, thus highlighting training and educational needs, which in turn should also motivate staff. Employing EQA to check whether laboratory performance meets specified requirements also increases customer confidence and the credibility of the IVF unit placing it in a more competitive position with its counterparts.

WHAT COMPRISES A QC PROGRAM?

A QC program involves the monitoring of: (i) equipment; (ii) culture media and consumables; (iii) the environment; and (iv) protocols. When setting up a new IVF unit or introducing new changes to an already established laboratory, validation of any new variables is required, which once established to be satisfactory should then be regularly monitored. All testing, whether it is the initial validation or routine monitoring, should take place at an adequate frequency using appropriate, accurate, reliable, and preferably accredited testing methods. Furthermore, all aspects of the testing should be accurately documented.

Equipment Validation

All new and major items of equipment (e.g., flow hoods, incubators, etc.) should be installed by the manufacturer and properly commissioned with certification. Even then, internal validation using QC measures of the equipment before its use in clinical practice is highly recommended to ensure that it meets the manufacturer's specifications (6). All new major items of equipment should be switched on monitored regularly allowing sufficient time for performance validation to ensure that problems are clearly identified and rectified before use in clinical treatment. The time required to validate an item of equipment will vary depending on its function. In setting up the ACU, CRMF in Sheffield, a period of 2 months was allowed to run and test new major items of equipment after their installation before employing them in clinical practice (6). As a result, problems were identified and solved with major items of equipment that otherwise would not have been detected if testing had lasted only a relatively short period of time.

Table 1 summarizes the types of equipment tested in situ within the ACU, CRMF, the type of test used and the frequency of testing. In addition to the tests listed, a mouse embryo bioassay was also used to assess the incubators before use in clinical treatment for their ability to support mammalian embryo development. Although the mouse embryo bioassay is generally considered a biologically relevant bioassay (7), it has been criticized for being insufficiently sensitive (8). However, its sensitivity can be manipulated by taking into account the strain of mouse used, the stage of embryo retrieval and culture, and the type of culture medium used (4,9). To test the three incubators in the ACU, CRMF, mouse 2-cell embryos of the MF-1 strain were randomly divided between these and a fourth (control) incubator in which successful mouse embryo development had previously been established. The control incubator was located in a separate research laboratory in the hospital within the Academic Unit of Reproductive and Developmental Medicine, University of Sheffield. Groups of 50 embryos were cultured in 50 µL microdroplets of KM3 medium (10) overlaid with liquid paraffin oil at 5% CO_2 in air and 37°C (11). Embryo

Table 1 Internal Quality Control Measures Used to Assess the Performance of New Equipment Installed in the Assisted Conception Unit, Centre for Reproductive and Developmental Medicine, Sheffield, U.K.

Equipment	Manufacturer	Model	Test	Frequency of testing	Method	Range ± accuracy
Incubator	Heraeus	BB6220	CO_2 (%)	Weekly	Fyrite gauge	5 ± 0.5%
			Temperature	Daily	Max/min thermometer	−10°C to +50 ± 1°C
			Environment	As required	Mouse bioassay	n/a
Water system	Vivendi	Purelab Maxima	Endotoxin	Weekly	Limulus Amoebocyte Lysate test	0.03 EU/mL
Refrigerator	LEC	LR500	Temperature	Daily	Max/min thermometer	−10°C to +50 ± 1°C
Heated stages	MAT[a]	BioMAT2	Temperature	Monthly	Surface thermometer	−50°C to +150 ± 0.1°C
	Hunter[a]	Hunter N24	Temperature	Monthly	Surface thermometer	As above
	Hunter	200AIN	Temperature	Monthly	Surface thermometer	As above
Hot blocks	Grant	QBT2	Temperature	Monthly	Mercury thermometer	−20°C to +150 ± 5°C
Hot oven	Gallenkamp	OVB-307	Temperature	Every use	Temperature indicator strips	121°C to 160 ± 2%
Cryopreservation equipment	Planar	Kryo 360	Function	Every use	Test run with medium only	n/a

[a]Integral to class II flow hoods.
Source: From Ref. 6.

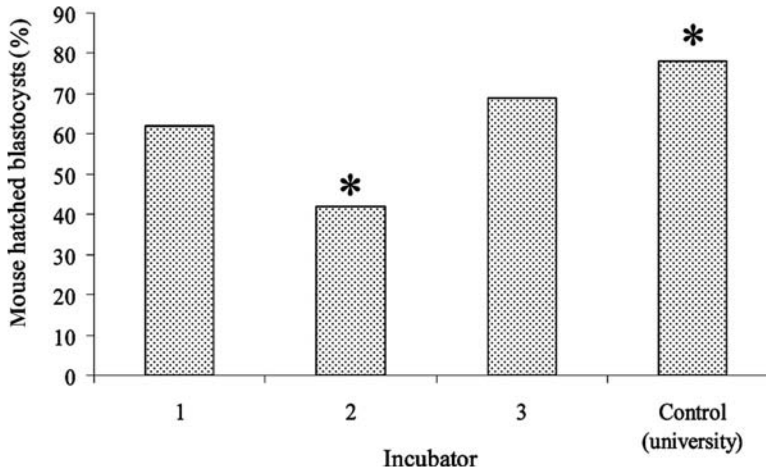

Figure 1 The percentage of 2-cell mouse embryos of the MF-1 strain reaching the hatched blastocyst stage in three new incubators newly installed in the Assisted Conception Unit, Centre for Reproductive Medicine and Fertility, Sheffield, U.K. and a fourth, control incubator located in the Academic Unit of Reproductive Medicine and Fertility, University of Sheffield. $*p < 0.05$. Fisher's exact test. *Source*: From Ref. 6.

morphology and cleavage rates were monitored daily over a period of 5 days and the percentage of embryos reaching the blastocyst stage on day 5 post-fertilization compared using Fisher's exact test (Fig. 1).

Using the QC measures outlined in Table 1 and the mouse embryo bioassay, several problems were identified with the new equipment installed and commissioned by the manufacturers in the ACU, CRMF, including the incubators, refrigerators, and the water purification system (6). The temperature of two of the incubators took more than ten weeks to stabilize at 37°C, whereas the third incubator exhibited unacceptable fluctuations even after this period of time (Fig. 2). Furthermore, the CO_2 levels in the third incubator also failed to stabilize satisfactorily (Fig. 3). The sub-optimal performance of the third incubator was confirmed by the mouse embryo bioassay. The proportion of mouse embryos reaching the hatched blastocyst stage using this instrument was significantly lower than that achieved by the control incubator ($p < 0.05$, Fisher's exact test; Fig. 1). The QC measures also identified a problem with two of the refrigerators which both failed to maintain the correct temperature for storing embryo culture medium (i.e., 2–8°C; Medicult, U.K.). The temperature of both refrigerators was consistently between 8 to 10°C. The ultra-pure water tested positive for endotoxins (> 0.03 EU/mL) on two separate occasions. Sanitization was carried out immediately according to the manufacturer's instructions and the equipment re-tested. On both occasions, the water tested negative following sanitization.

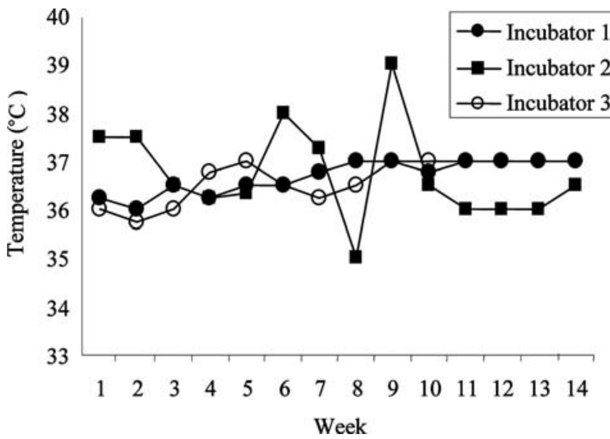

Figure 2 Temperature recordings from three incubators newly installed in the Assisted Conception Unit, Centre for Reproductive Medicine and Fertility, Sheffield, U.K. All readings were carried out daily using a using a maximum/minimum thermometer (−10°C to +50 ± 1°C). *Source*: From Ref. 6.

One of the main outcomes from this study was that independent double-checking and performance validation of new equipment before use in clinical treatment, even after installation and commissioning by the manufacturer, is very important.

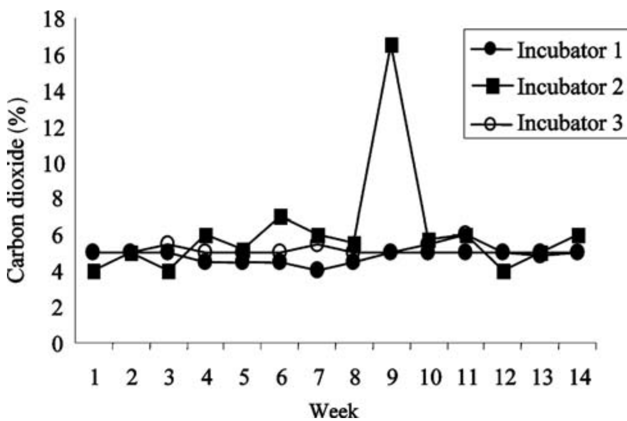

Figure 3 CO_2 recordings from three incubators newly installed in the Assisted Conception Unit, Centre for Reproductive Medicine and Fertility, Sheffield, U.K. All readings were carried out weekly using a fyrite gauge (−10°C to +50 ± 1°C). *Source*: From Ref. 6.

Equipment Monitoring

Following installation and validation, continuous and regular monitoring of the equipment once in clinical use is imperative to ensure its performance is maintained and that problems are detected as they occur. In the ACU, CRMF, independent temperature recordings of the refrigerators and incubators continued to be tested daily, the hot blocks and heated stages monthly, and the hot oven every time it was used; the CO_2 levels in the incubators were monitored weekly. The cryopreservation equipment was also assessed after each freezing run by checking that the printed readout detailing the embryo cryopreservation program followed the specified program details (6). Taking into account the manufacturers' recommendations on frequency of servicing, all equipment was also placed on a service contract (6). Regular servicing of items of equipment minimizes the risk of equipment malfunction and, although seemingly expensive at the outset, is usually cost-effective in the long-term.

Culture Media and Consumables Validation

Certification of product QC testing by the manufacturer should be obtained for all consumables used in the laboratory. Given the increasing wide choice and availability of culture media and other chemicals/solutions for human IVF, it is becoming less acceptable to make such consumables in-house unless they are rigorously assessed using QC. However, even when purchasing company-manufactured media, detailed inspection and critical evaluation of a company's QC program is essential to ensure that it meets appropriate standards for IVF. For example, some manufacturers lay claim to using the mouse embryo bioassay to test their products. However, their definition of cytotoxicity may be <40% blastocyst development using an F1 hybrid or inbred strain. This is a very low cut-off point which may not identify all embryo toxic products. Furthermore, proof of testing upon delivery of each new batch of consumables/culture media is not always forthcoming (4). Once again, QC of new consumables, even those already QC-tested by the manufacturer, is recommended before their use in the IVF laboratory to ensure their suitability. This is especially applicable to those consumables not specifically designed for use in IVF (e.g., gloves, some cell culture flasks, and dishes) which may be suitable for culturing other types of mammalian cells but may prove to be embryo toxic (12).

A useful method for testing consumables is the human sperm survival assay (13). This is an inexpensive and convenient bioassay, not involving the use of animals, which monitors sperm survival over hours/days in culture while in contact with the products. As with the mouse embryo bioassay, consideration must be given to the conditions under which the assay is performed to ensure appropriate sensitivity to detect cytotoxicity of clinical significance. Claassens et al. (14) found that optimum sensitivity was

achieved when sperm were cultured in the absence of serum. A calculated sperm motility index value of < 0.75 was used to indicate sperm toxicity, and items identified as sperm-toxic within eight hours were considered to be of clinical significance due to close agreement with the mouse embryo bioassay (14). Validation of consumables before use in clinical treatment is important to ensure that an alternative product can be sourced should they prove to be unsuitable. If an alternative product needs to be found then sperm survival tests should be repeated as appropriate.

Culture Media and Consumables Monitoring

Despite QC testing by the manufacturer and initial validation of a product, QC testing of all new batches of culture media is important because transport and incorrect storage conditions can affect media quality (4). The QC measures should include testing for endotoxin levels because their presence in embryo culture medium can cause a significant decrease in pregnancy rates (15). The limulus amoebocyte lysate test is a simple and rapid method for checking endotoxin levels in company-manufactured media as well as the water of any in-house water purification system (6). The osmolarity of all new batches of culture medium should also be checked to ensure that it is within the acceptable range based on the manufacturer's information.

Similarly, all new batches of plastics and other consumables should be subject to a sperm survival test because seemingly minor changes in the manufacturing process can affect the quality of the product rendering it embryo-toxic. Moreover, the manufacturers sometimes fail to report changes in specifications/manufacturing process to end users.

The batch / lot numbers for all consumables and media to come into contact with gametes and embryos should be documented, preferably in each patients' records, to ensure comprehensive tracing of every product should a problem arise (4).

Environment Validation

Involvement in the planning and design of a new IVF unit, or building/ decorating alterations to an existing unit, can save a lot of trouble further down the line. At the first instance, consideration should be given to possible sources of indoor air contaminants because volatile organic compounds (VOCs) and chemical airborne contaminants (CACs) can significantly affect mouse embryo culture (2). Factors affecting the levels of internal air pollutants include the air quality outside the building, equipment, heating, ventilation and air-conditioning systems, human activities, building components and furnishings, and other temporary sources such as redecorating and repair activities. When possible, consideration should therefore be given to the site and location of the laboratories. The use of air purification units such as the CODA® towers and positive pressure airflow in the laboratories

should also be considered, if necessary, to reduce the levels of airborne contaminants. To minimize the levels of VOCs and CACs when setting up the ACU, CRMF, sealants and toxic glues were avoided, and low odor specialized paint was used for decorating the laboratories (6). The quality of the gas supplying the incubators and the type of pipework used should also be carefully considered. Class II biological safety cabinets with High efficiency particulate air-filtered air, which provide personnel as well as environmental and product protection, were also installed for all gamete and embryo handling. The positioning of air conditioning or purification units should be carefully considered so that the airflow of the class II biological cabinets is not compromised.

Laboratory lighting should also be taken into account at the planning stage because ultraviolet (UV) light may have detrimental effects on mammalian oocyte maturation and embryo development (16,17). Tungsten light bulbs controlled by dimmer switches can be installed to provide low-level lighting in the laboratories (6). Alternatively, UV light filtration units can be used to filter out harmful UV wavelengths on fluorescent lights.

Once building works/decorating is completed, microbiology testing of the air quality by an accredited laboratory is recommended. At the ACU, CRMF, swabs and settle plates were used to test the presence of fungus or colony-forming units (CFUs) on laboratory surfaces and in the incubators and flow hoods (6). Acceptable limits of air quality for a clean area were considered to be <100 CFUs and no fungus (Department of Microbiology, Royal Hallamshire Hospital, Sheffield, U.K.) and less than one CFU and no fungus within the class II flow cabinets (18). Initial validation of the air quality within the new laboratories before treatment commenced was found to be satisfactory (Fig. 4—August). Similarly, no bacterial growth was

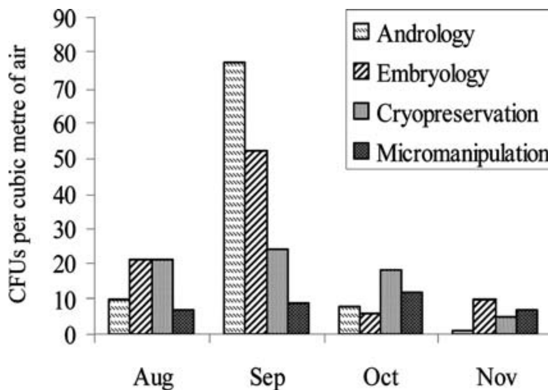

Figure 4 Microbiological testing of air quality in the in vitro fertilization laboratories of the Assisted Conception Unit, Centre for Reproductive Medicine and Fertility, Sheffield, U.K. *Abbreviation*: CFUs, colony-forming units. *Source*: From Ref. 6.

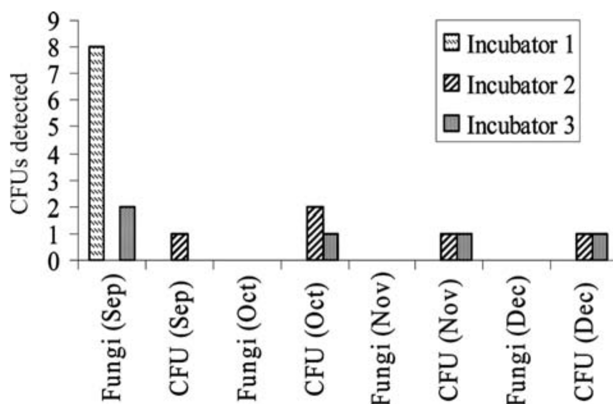

Figure 5 Microbiological testing of three incubators newly installed in the Assisted Conception Unit, Centre for Reproductive Medicine and Fertility, Sheffield, U.K. *Abbreviation*: CFUs, colony-forming units. *Source*: From Ref. 6.

observed in the initial validation of the water from the in-house water purification system. However, microbiological testing revealed that fungi were present in two of the three incubators (Fig. 5), both of which were humidified. The third incubator was non-humidified and had no infection. Therefore, after thorough cleaning and retesting, protocols were reviewed and all three incubators were run non-humidified. Tests were carried out to ensure that the osmolarity of the culture medium overlaid with oil was stable for >2 days (6).

The safety and security of gametes and embryos as well as that of staff working in the laboratories is also important. Alarm systems can be installed to detect a number of variables including liquid nitrogen levels in gamete and embryo storage dewars, environmental oxygen levels in the cryopreservation laboratory, and pressure in the pipes supplying CO_2 to the incubators (6). All alarms should be validated prior to use and subject to QC and monitoring once in use. All alarms should be linked to a central control system which will alert staff to any fault 24 hours a day.

Environment Monitoring

Regular monitoring of the environment in the established laboratory is important to ensure that appropriate standards are maintained (2,19). Microbiology and bacteriology testing, as outlined above at ACU, CRMF, was carried out every month once treatment commenced. This regularity of testing revealed a high number of CFUs in the laboratory air in the month of September and was attributed to an engineer carrying out equipment maintenance the day before the air was sampled (Fig. 4). As a result, laboratory protocols were amended to include a weekly rota for cleaning the

laboratories and an additional cleaning procedure after any maintenance or servicing had been carried out.

Other environmental parameters should also be closely monitored including laboratory temperature and if possible levels of VOCs and CACs.

WHAT COMPRISES A QA PROGRAM?

QA includes QC as well as a number of other measures such as those listed below. This list is by no means comprehensive but illustrates what QA measures can be employed to monitor and improve laboratory performance, and thus improve patient care.

Treatment Monitoring

Validation of Laboratory Performance in a New IVF Unit

Before commencing IVF in a new center, other types of treatment such as intrauterine insemination (IUI) can be started and monitored to assess some aspects of laboratory practice, including protocols and consumables. For example, at the ACU, CRMF, the clinical pregnancy rate of 40 cycles of stimulated IUI was monitored before IVF treatment was started. A 35% clinical pregnancy rate (fetal heart detected per treatment) was achieved after 40 cycles suggesting no negative effect on sperm function following gamete preparation in the Andrology Laboratory.

Monitoring of Laboratory Performance in an Established IVF Unit

Performance of the IVF laboratory can be monitored by auditing fertilization and embryo cleavage rates and embryo quality at an appropriate frequency depending on the center's activity (20). The pregnancy rate, implantation rate, and multiple birth rates can also be audited. However, when interpreting these statistics, consideration must be given to the activity of the unit and the types of patients treated, etc (1). Furthermore, pregnancy rate alone should not be used as a QC measure to audit the performance of the laboratory. This is not least because it is affected by other variables, but also because a drop in pregnancy rate identifies a problem encountered at the time of treatment usually weeks before. A good QC program detects problems before they affect the pregnancy rate. Moreover, using pregnancy rate as a QC measure does not identify the source of the problem (4).

Record Keeping and Documentation

Fundamental to the success of an QA program is accurate documentation and record keeping of all aspects of the scheme. In designing the paperwork,

thought should be given to information retrieval, traceability, and security of the documents. The option of using computer records should also be considered. Systems should also be in place to ensure that information is accurately recorded when transferred from one format to another. All standard operating procedures and protocols should also be documented and updated frequently, and made readily available to all staff.

With regards to the IVF laboratory, the monitoring of gametes and embryos from the time of collection to treatment outcome should be documented thoroughly together with accurate recording of patient information. A system should be in place to double check that information is accurately recorded during laboratory procedures, particularly when it comes to identifying patients' gametes and embryos. In the United Kingdom, under the regulation of the Human Fertilization and Embryology Authority, it is now obligatory that all laboratory procedures involving the manipulation and transfer of patient's gametes and embryos are witnessed and documented by a second member of staff.

Incident Reporting

However minor they seem at the time, all incidents/events should be reported and documented as soon as possible as they may lead to an unforeseen effect on laboratory performance and/or treatment outcome. For example, recording the servicing of a flow cabinet enabled laboratory staff of the ACU, CRMF, to account for an unexpected increase in CFUs detected in the air quality (6).

Appraisal of Personnel Performance

EQA can be used to appraise the performance of laboratory staff in various procedures. For example, the U.K. National External Quality Assessment Service (NEQUAS; www.unequas.org.uk) offers schemes for assessing various andrology investigations including semen analysis, sperm antibody, and sperm motility testing. Unfortunately, there are few national and even fewer international accredited EQA schemes for other procedures used within the IVF laboratory. However, internal assessments can be devised to appraise the performance of personnel in methods such as therapeutic embryo grading (5).

The primary purpose of regular staff evaluation is to identify areas for improvement and further training. All staff should undergo regular appraisals with their head of department and all skills and training of staff accurately documented (1). Training should preferably be certified according to a recognized body such as the Association of Clinical Embryologists (ACE) in the United Kingdom, which offers two types of programs, the Certificate and the Diploma, both of which are carried out in-house but are assessed externally (21).

INTERNATIONAL STANDARDS OF QA AND QC

Given the advantages of employing the QA and QC measures outlined above, why has it taken so long for the IVF laboratory to routinely employ these schemes? Reasons include the lack of appropriate and specific standards with which to comply, and regulatory authorities to enforce them. Time and money also need to be invested at the outset to implement and then run QA and QC programs. To date, self-motivation has been the major factor driving units to employ QA and QC schemes; however, legislation is now increasingly putting pressure on IVF units to employ such program.

On a national level, evidence of QA and QC in the IVF laboratory is becoming a requirement in some countries with various inspecting authorities such as the Human Fertilisation and Embryology Authority (HFEA) in the United Kingdom, and the Society for Assisted Reproductive Technology (SART) in the United States. Furthermore, IVF units within the European Union (EU) will have to comply with the EU Tissues and Cells Directive by April 2006, which requires units to have QC and QA systems. Some countries also now have governing bodies that can evaluate and certify IVF laboratories. In the United States, there are various organizations that offer this service including the Joint Commission on Accreditation of Healthcare Organizations and the College of American Pathologists. For those countries without national accrediting bodies, there are guidelines available for IVF laboratories issued by recognized societies within the field such as the ACE standards and guidelines for IVF laboratories (21) and the ESHRE guidelines for good practice in IVF laboratories (22), which include recommendations for QA and QC. However, these guidelines are not obligatory and are usually too generalized. The importance of having internationally recognized standards for QA and QC is therefore now being realized (1).

The International Standards Organisation (ISO) is a non-governmental organization and the world's largest developer of international standards (23) It is currently comprised of a network of national standard institutes from 148 countries, with one body representing each country and a central secretariat based in Geneva, Switzerland, which coordinates the system. Its purpose is to set internationally recognized standards to enhance quality, increase reliability, and improve health and safety of products and services world-wide.

The first European IVF unit to apply for internationally recognized accreditation was the Fertility Centre of Scandinavia, Goteberg, Sweden, which applied for ISO/IEC 17025 (formerly ISO/IEC Guide 25). These are standards jointly devised by ISO and its international partner, the International Electrotechnical Commission General, which defines Requirements for the Competence of Testing and Calibration Laboratories (4). The ISO 9000 series, more specifically ISO 9001:2000, is now the more common standard applied for by IVF units. ISO 9001:2000 specifies "requirements for a

quality management system for any organization that needs to demonstrate its ability to consistently provide a product or service that meets customer and applicable regulatory requirements, and thereby aims to enhance customer satisfaction.'' Thus, conformance to ISO 9000:2000 is said to guarantee that a company delivers quality products and services.

To apply for ISO 9000:2000 an IVF unit's management team initially needs to define and formally document its QA policies and objectives and how these will be implemented. An external consultant can be employed to assist in this process. Once the documentation is in place and the QA measures have been implemented, an external assessor examines the unit's QA system to ensure it complies with ISO 9000:2000. A detailed report is then submitted describing sections of the standard that the unit has failed to comply with. The unit is then given a specified time limit in which to correct the problems. Once these have been addressed, the unit is certified as in conformance with the standard.

The benefits of having internationally recognized standards are several fold (1). They include overcoming differences between national standards, thus allowing comparisons between laboratories in different countries. Furthermore, the use of internationally recognized practices should improve the standards and therefore the success rates of IVF. As the benefits of internationally recognized QA and QC schemes are now being realized, the number of ISO-accredited IVF units is on the increase. This can only serve to benefit the reputation of IVF as a clinical science but also the patients for whom they care.

REFERENCES

1. Alper MM, Brinsden PR, Fischer R, Wikland M. Is your IVF programme good? Hum Reprod 2002; 17:8–10.
2. Hall J, Gilligan A, Schimmel T, Cecchi M, Cohen J. The origin, effects and control of air pollution in laboratories used for human embryo culture. Hum Reprod 1998; 13:146–151.
3. Gardner DK, Lane M. Embryo culture systems. In: Trounson AO, Gardner DK, eds. Handbook of In vitro Fertilization. Boca Raton: CRC Press, 1999:205–264.
4. Wikland M, Sjoblom C. The application of quality systems in ART programs. Mol Cell Endocrinol 2000; 166:3–7.
5. Matson PL. Internal quality control and external quality control assurance in the IVF laboratory. Hum Reprod 1998; 13(suppl 4):156–165.
6. Cutting R, Pritchard J, Clarke H, Martin K. Establishing quality control in the new IVF laboratory. Hum Fertil 2004; 7:119–125.
7. Quinn P, Horstman F. Is the mouse a good model for the human with respect to the development of preimplantation embryo in vitro? Hum Reprod 1998; 13(suppl 4):173–181.
8. Rinehart JS, Bavister BD, Gerrity M. Quality control in the in vitro fertilisation laboratory: comparison of bioassay systems for water quality. J In Vitro Fertil Embryo Transf 1988; 5:335–342.

9. Zarmaoupis-Zavos PN, Zavos PM. Factors that may influence the mouse embryo bioassay. Tohoku J Exp Med 1996; 179:141–149.
10. Morris KA, Martin KL. Effects of activin A on embryo development during implantation in vitro [abst]. Reproduction 2002; 28:35.
11. Martin KL, Leese H. Role of glucose in mouse preimplantation embryo development. Mol Reprod Dev 1995; 40:436–443.
12. Reddy VR, Thomas TS, Wright HR, et al. The scientific basis of surgical glove selection in an in vitro fertilisation laboratory. J Biomed Mat Res 1999; 48(4):569–571.
13. Critchlow JD, Matson PL, Newman MC, et al. Quality control in an in vitro fertilisation laboratory: use of sperm survival studies. Hum Reprod 1989; 4: 545–549.
14. Claassens OE, Wehr JB, Harrison L. Optimizing sensitivity of the human sperm motility assay for embryo toxicity testing. Hum Reprod 2000; 15:1586–1591.
15. Snyman E, Van der Merwe. Endotoxin-polluted medium in a human in vitro fertilisation program. Fertil Steril 1986; 46:273–276.
16. Bradshaw J, Jung T, Fulka J Jr., et al. UV irradiation of chromosomal DNA and its effect upon MPF and meiosis in mammalian oocytes. Mol Reprod Dev 1995; 41:503–512.
17. Evans J, Wells C, Hood K. A possible effect of different light sources on pregnancy rates following gamete intra-Fallopian transfer. Hum Reprod 1999; 14:80–82.
18. Department of Health: A code of practice for tissue banks, providing tissues of human origin for therapeutic purposes. Medicines Control Agency, Department of Health, ISBN 1 841823295, 2001.
19. Cohen J, Gilligan A, Esposito W, Schimmel T, Dale B. Ambient air and its potential effect on conception in vitro. Hum Reprod 1997; 12:1742–1749.
20. Balaban B, Urman B. Embryo culture as a diagnostic tool. Reprod Biomed Online 2003; 7:671–682.
21. (www.ace.ivf.net).
22. Gianaroli L, Plachot M, van Kooij R, et al. ESHRE guidelines for good practice in IVF laboratories. Committee of the special interest group on embryology of the European Society of Human Reproduction and Embryology. Hum Reprod 2000; 15:2241–2246.
23. (www.iso.org).

18

Oocyte Donation: State of the Art

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INTRODUCTION

Oocyte donation is now an integral part of the armamentarium of the infertility specialist. At its inception, it was intended as therapy for women with premature ovarian failure, or those with heritable genetic defects. However, as experience accumulated, oocyte donation as a treatment has been extended to women with a variety of defects in oocyte production or function. The largest group of women now undergoing treatment with oocyte donation consists of those with age-related decline in fertility. Additionally, techniques learned from oocyte donation, including endometrial preparation and embryo-endometrial synchrony, have been applied to other infertility treatments, including frozen embryo transfer and in vitro maturation of immature oocytes.

HISTORY

The first successful human oocyte donation was reported in 1983. At the time of ovulation, a fertile donor was inseminated with the recipient partner's sperm. Uterine lavage was performed on the fifth day after the luteinizing hormone (LH) peak. The recovered embryo was then transferred to the uterus of the infertile patient. After several attempts, a pregnancy was successfully initiated and a singleton birth at term was achieved (1,2).

Unfortunately, this method, known as "ovum transfer," was inefficient (3). Attempts to enhance the efficiency of the process by administering fertility medications to the donors were unsuccessful and led to retained pregnancies in the donors (4).

The first use of standard in vitro fertilization (IVF) methodology for oocyte donation was also reported in 1983; however, this pregnancy ended as a miscarriage (5). Subsequently, Lutjen et al. reported a successful pregnancy in a woman with ovarian failure. Donated oocytes were fertilized in vitro and the resulting embryos were transferred to the recipient's uterus, which was prepared with a combination of oral estradiol and intramuscular progesterone (6). This landmark event substantiated the observation that exogenous estrogen and progesterone could reliably produce a receptive endometrium rather than relying on natural ovulatory cycles. In women with residual ovarian function, donor and recipient cycles could thus be synchronized with gonadotropin-releasing hormone (GnRH) agonists (7), and the recipient endometrium stimulated with exogenous steroids (8,9). These essential principles have remained the key components of the current practice of oocyte donation.

INDICATIONS

Oocyte donation is a therapy which allows women to conceive when their own oocytes are either not capable of producing a pregnancy or carry a heritable defect that the patient does not want to pass on to their children. Common indications for oocyte donation thus include premature ovarian failure secondary to chemotherapy in healthy cancer survivors (10), follicle-stimulating hormone (FSH) receptor defects (11), or other causes. Patients with gonadal dysgenesis are also appropriate candidates for oocyte donation. Those with Turner's syndrome may conceive; this group may be at some risk of obstetrical complications (12). Oocyte donation is also indicated in the cases of poor oocyte quality inferred by multiple failed cycles of conventional IVF or in women of advanced reproductive age with associated diminished ovarian reserve. Other patients may choose oocyte donation in an attempt to avoid passing heritable genetic diseases to their children, although pre-implantation genetic diagnosis is increasingly being applied in these situations.

FACTORS INFLUENCING SUCCESS

Donor Age

It has been established that female fertility hinges on the age of the oocyte provider. Thus, the chronological age of the oocyte donor would seem to play an intuitive role in the overall success of the process. Remarkably, most

retrospective series do not demonstrate a major effect of donor age on pregnancy success (13–18). However, retrospective series suffer from selection bias; older donors may indeed have been chosen for inclusion because of their prior good track record. Some studies have confirmed that older donors may be less likely to produce a clinical pregnancy (19–21). Although prior fertility in a donor does not appear to play a role (19,21), previous successful cycles may improve the prognosis in subsequent ones (15).

Recipient Age

Recipient age per se does not appear to decrease the probability of pregnancy success with oocyte donation. Indeed, it was the observation that the age of the uterus does not influence the probability of the initiation of pregnancy that led to the conclusion that the age-related decline in human fertility was primarily due to the age of the oocyte (22–27). Supporting this contention were physiological observations that uterine blood flow does not appear to decrease with age (28) and that the endometrial response to exogenous steroids also does not appear to diminish with age (29). It is not yet definitively established whether pregnancy success after the age of 50 remains unaltered. In our experience (30,31), no decline has been observed. However, an analysis of data derived from the national registry suggested a modest decline in live birth rates after 50 years of age (32).

Maternal Factors

The specific indication for oocyte donation does not appear to influence the success of the procedure (15,27,32). However, uterine pathology, such as Asherman's syndrome or previous radiation therapy, may negatively impact uterine receptivity (33). Analogously, uterine fibroids or other endometrial lesions are thought to interfere in embryo implantation, regardless of whether or not oocyte donation is used (34). It should be noted that the incidence of uterine pathology increases with the chronological age of the recipient. Therefore, older recipients may require more careful screening for these factors. Hydrosalpinges in recipients also appear to negatively impact embryo implantation (35), and thus we recommend imaging of the fallopian tubes in all potential recipients. Obesity in recipients does not appear to impact pregnancy rates (36,37); one report suggested an increase in miscarriage rates (38). Endometriosis in recipients does not negatively impact embryo implantation (39). Pinopod expression, a marker of uterine receptivity, appears to be the same in recipients with endometriosis as in those without the disease (36).

Repetitive Cycles

Lack of success in one or more cycles does not negatively impact the outcome in subsequent cycles, and pregnancy rates appear to remain unaltered

on a per cycle basis (32) as long as the recipient has a normal uterine cavity and normal endometrial development was verified. However, in one series, patients who conceived during their first cycle of oocyte donation were more likely to conceive in subsequent cycles (40).

Cycle fecundability also appears to be unaltered by the number of cycles that the donor has undergone. The donor response to gonadotropin therapy and pregnancy rates do not differ by the interval between donation cycles or the cycle rank (41,42). Donor complications are uncommon and do not appear related to the number of stimulation cycles. Nevertheless, the American Society for Reproductive Medicine (ASRM) recommends that an individual limit the number of donor cycles to no more than six (43).

SCREENING OF DONORS

The ASRM publishes and periodically updates its guidelines for donor screening (44). Screening guidelines include a personal and sexual history, with the intent of excluding those women at high risk for HIV, other sexually transmitted disease, transmissible spongiform encephalopathy (44), or recent confirmed or suspected West Nile virus infection (45). Serum screening for syphilis, hepatitis B and C, HIV-1, and HIV-2, as well as cervical cultures for Neisseria Gonorrhoea and Chlamydia, is recommended (44).

Psychological screening should include family, sexual, substance use, psychiatric history, information on educational background, assessment of stability, motivation to donate, life stressors and coping skills, and interpersonal relationships. The counselor should confirm that the donor has been informed of the medical treatment, discuss the psychological risks of oocyte donation, and evaluate for signs of coercion (46).

Genetic screening should include a history with a specific focus on any major Mendelian disorders, major malformations due to multifactorial causes, or known karyotypic abnormalities in the potential donors or in any of their first-degree relatives (47). A screening test for cystic fibrosis is recommended for all donors. A heterozygote may be included as a donor if the recipient is aware and the recipient's partner tests negative (48). Specific ethnic groups known to be at high risk for carrying certain recessive genes should undergo additional screening tests (47).

SCREENING OF RECIPIENTS

As with donors, the ASRM periodically updates its guidelines for the screening of recipients (44). Routine screening includes a medical and reproductive history, physical exam, and standard preconception testing and counseling. These include blood type and Rh factor, rubella and varicella titers (with vaccination if not immune) along with infectious disease screening. Recipients

should undergo counseling regarding the potential psychological implications of becoming parents as a result of oocyte donation (46).

The uterus should be examined by ultrasound and the cavity evaluated and pathology corrected prior to embryo transfer (36). Evaluation of the endometrial cavity can be achieved by saline-injection sonography (49), hysteroscopy, or hysterosalpingography (HSG). The advantage of the HSG lies in its ability to visualize the fallopian tubes and thus detect the presence of hydrosalpinges. All hydrosalpinges should be removed, as these have been shown to decrease implantation rates and increase the risk of infection as well as ectopic pregnancy in recipients of oocyte donation (50,51). If salpingectomy is not possible, interruption of the oviduct in a location proximal to the uterotubal junction also appears to prevent reflux of hydrosalpingeal fluid and thus to mitigate the adverse effects of the hydrosalpinx (52). As a special group, patients with Turner's syndrome should undergo echocardiography because they are thought to have as high as a 2% risk of aortic rupture or dissection with a risk of death during pregnancy (12).

The screening of partners of recipients of oocyte donation should include a semen analysis, blood type and Rh screen, and a genetic screen along with infectious disease screening for syphilis, hepatitis B and C, cytomegalovirus antibody, and HIV. Screening should also include psychological counseling.

PRACTICE CYCLE

When oocyte donation first began, all recipients underwent practice cycles to ensure an adequate response of the recipient's endometrium to exogenous steroids. Although this practice has recently become somewhat controversial in that some programs feel that it is not necessary, our program continues to rely on the information the cycle provides. In a practice cycle, recipients undergo a regimen of exogenous estrogen and progesterone, which is identical to the actual recipient cycle. Our practice utilizes the regimen depicted in Table 1. Endometrial thickness is noted at the beginning of the cycle and prior to the initiation of progesterone. On the seventh day of progesterone administration, endometrial thickness is again documented and the recipient undergoes a practice embryo transfer as well as endometrial biopsy. We have found that ultrasound measurement of endometrial thickness does not always correlate with biopsy findings (54). The biopsy may the document a lack of adequate estrogen priming or out-of-phase endometrium (55). Practice cycles also provide additional information on patient's ability to comply with the hormone therapy protocol. In one large study, 5.8% of women had difficulty in following the instructions (54). A recent report has suggested that local injury to the endometrium during non-transfer cycles may improve subsequent implantation rates during IVF, although the mechanism is not clear (56).

Table 1 Standard Estrogen and Progesterone Replacement Regimen

	Micronized estradiol (oral administration)	Micronized progesterone (vaginal administration)
Days 1–4	1 mg BID	
Days 5–9	2 mg BID	
Days 10–14	2 mg TID	
Days 15–28 (through 13th week of gestation)	2 mg BID	200 mg TID

Source: From Ref. 53.

In contrast, programs that no longer require endometrial biopsies rely on information from studies indicating that an in-phase endometrial biopsy does not predict pregnancy (57) and an out-of-phase biopsy does not necessarily predict failure (58). Pregnancy rates in one small study did not differ whether a biopsy was or was not performed (57). It has also been argued that measurement of the endometrial thickness may be a better predictor of outcome (58) and one study did find that it correlates with biopsy (59).

OVARIAN STIMULATION

Oocyte donors undergo controlled ovarian hyperstimulation in a manner similar to that of conventional IVF. The most common method is the so-called "long protocol," which relies on pre-stimulation pituitary downregulation using GnRH agonists in daily intermittent or depot formulations. More recently, short protocols using GnRH antagonist during the late follicular phase of the stimulation cycle have been utilized. Studies comparing agonists and antagonists have not shown a significant difference in pregnancy rates achieved with protocols using agonists or antagonists (60,61). As with conventional IVF, the use of antagonists appears to reduce the amount of gonadotropins required and the duration of treatment (60,62). When utilizing antagonists, gonadotropin dosage may need to be adjusted to prevent a potential decline in estradiol levels, which has been correlated with decreased pregnancy and implantation rates (63). Others have advocated adding recombinant LH to recombinant FSH protocols, when starting antagonists, as a strategy to increase oocyte yield and improve pregnancy rates (64).

In agonist cycles, controlled ovarian hyperstimulation follows pituitary downregulation in the standard fashion as per conventional IVF using recombinant or purified gonadotropins. The addition of recombinant LH to recombinant FSH in donors on agonists with an LH value of less than

1 IU/L during downregulation may increase the number of mature oocytes and the implantation rate (65). Dosages are individualized based on donor characteristics. For example, non-hirsute fertile women with polycystic-appearing ovaries may require fewer ampoules to meet criteria for oocyte retrieval compared to donors without polycystic-appearing ovaries; fertilization rates tend to be similar (66). During the stimulation phase, serum estradiol levels on day 5 (67) and at peak (68) have been reported to correlate with the number of oocytes as well as with the implantation rate.

In our practice, donors are most commonly prescribed an overlapping regimen of oral contraceptives and GnRH agonists in a long protocol. Oral contraceptives are begun one week before the initiation of leuprolide acetate. Both medications are continued for one week, and the oral contraceptives are then stopped, whereas leuprolide is continued. Oral contraceptives allow for coordination of recipient and donor cycles. Donors are evaluated approximately one week after cessation of oral contraceptives prior to stimulation to confirm downregulation by ultrasound evaluation of the ovaries and endometrium and by measurement of serum estradiol. Stimulation is then performed using human menopausal gonadotropins alone or in conjunction with recombinant FSH.

Donor cycles require vigilance on the part of the nursing staff. One study showed that despite thorough and repetitive instructions, 2% of donors were found to have difficulties adhering to the protocol and 7% become pregnant after stimulation, despite instructions to use barrier methods of contraception (69). We now advise donors to abstain from intercourse from the onset of stimulation until the following menses. In addition, donors are given a prescription for oral contraceptives to initiate with menses.

DONOR SAFETY

Serious complications of oocyte donation are relatively rare. Less than 1% of donors have events requiring hospitalization or emergent intervention during or following aspiration as a result of any cause, including severe ovarian hyperstimulation syndrome (OHSS), reactions to anesthesia, or intra-abdominal bleeding (70). Severe OHSS is uncommon (71); however, mild forms should be anticipated as a majority of donors experience abdominal discomfort and bloating to some extent. The symptoms usually resolve by the time of menstrual flow. As the severity of OHSS is correlated with estradiol levels on the day of human chorionic gonadotropin, overstimulation of donors should be avoided (72). Other general principles in the prevention and management of OHSS apply in donors as in infertility patients with the significant advantage that donors do not become pregnant and thus avoid pregnancy-related exacerbation of OHSS (73). Additional intravenous fluid and/or albumin may be administered at the time of follicle aspiration (74). In our practice, donors are evaluated in our office two days

after follicle aspiration. If necessary, additional intravenous fluids (1000–2000 mL of normal saline) may be administered. Rarely, paracentesis may be required (75). A small retrospective study showed that "coasting" in an attempt to prevent OHSS does not affect pregnancy rates unless coasting lasts for more than four days (76).

Little is known about the effect of oocyte donation on future health and fertility. However, in a study of eight donors who each underwent 4 to 12 cycles, laparoscopy confirmed no pelvic pathology except minimal endometriosis in one patient (77).

ENDOMETRIAL PREPARATION

The goal of endometrial preparation is to prepare the uterine cavity for implantation and to synchronize endometrial progress with embryo development. In general, preparation of the recipient endometrium with estrogen and progesterone occurs in fashion identical to the recipient practice cycle (Table 1). Women with ovarian failure can initiate a cycle at any time, whereas those with residual ovarian function should undergo pituitary downregulation with agonists to avoid untimely uterine bleeding associated with fluctuating steroid levels. Additionally, premature progesterone production by the recipients' ovaries can disrupt synchrony between embryos and endometrium. Using agonist therapy allows coordination of the oocyte donor and recipient and prevents ovulation, but does not appear to affect implantation rates (78). One study (79) suggested that downregulation in the recipient with residual function may not always be necessary. In that trial, 4 mg of oral estradiol daily starting on cycle day 1 suppressed ovulation (as determined by serum progesterone levels) until day 14 of the cycle in 97% of patients. Unfortunately, this regimen limits the flexibility for synchronization with donor stimulation in fresh cycles and is thus primarily used in frozen-thawed embryo transfer cycles.

Ever since Navot et al. showed that in-phase endometrium could be obtained with a variable estrogen and progesterone regimen, designed to mimic the natural cycle (80), variations on this combination have been used. To date, no study has demonstrated that a better endometrial environment can be generated by any other means. In other words, although many other biochemical substances are involved in the actual implantation process, their production is secondary to the steroid stimulation of the endometrial cells.

Estrogen stimulation can be achieved by a variety of regimens utilizing different routes of administration as well as different duration of administration. Estrogen may be delivered via an oral, transdermal, or vaginal route in a fixed or variable regimen. Oral or transdermal estradiol is most commonly used. In one study comparing a transdermal 0.1 mg patch to 6 mg of oral estradiol daily, less of a delay in glandular histology was noted despite lower serum estradiol levels (81).

Estradiol therapy can be provided in a fixed or variable regimen with comparable efficacy (82). Although fixed regimens using 1 mg of estradiol a day appear to be ineffective (82), fixed regimens using 4 or 8 mg/day without downregulation have been used to achieve an endometrial thickness greater than 6 mm in an average of seven days with good pregnancy rates. Navot et al. found that such small administration of fixed dosage estradiol compared favorably to a longer variable regimen (83); however, others have noted higher miscarriage rates with such short duration of estradiol therapy (84). In contrast, prolonged duration of estradiol therapy does not appear to have a negative impact (83,85).

Serum estradiol measurements during estradiol therapy appear to be unnecessary (27,86). However, multiple studies have shown that mid-cycle thickness correlates with pregnancy success (36,85,87). Although pregnancies occur in patients with a thinner endometrium measurement (88,89), it appears that endometrial thickness of $\geq$7 mm offers superior results. The presence of a multilayer endometrial echo complex may (90) or may not matter (91).

In patients who cannot achieve an adequate endometrial thickness with oral or transdermal estradiol alone, vaginal estradiol may be used. The vaginal route of steroid administration results in high endometrial tissue levels and these appear to increase the endometrial thickness (85,92). Low-dose aspirin (81 mg/day) has also been reported to increase the endometrial thickness (93). A combination of pentoxifylline 800 mg and tocopherol (Vitamin E) 1000 IU for nine months prior to hormone therapy has also been reported as therapy to enhance endometrial responsiveness in some women (94).

Following adequate estrogen priming of the endometrium, it is progesterone that prepares the endometrium for implantation. However, progesterone cannot act in the absence of adequate priming. Thus, an endometrial biopsy taken during a practice cycle, which shows no progestational effect has, in our experience, generally been reflective of inadequate estrogen priming rather than inadequate progesterone delivery to the endometrium. This effect may be observed in women after long episodes of amenorrhea, such as that occurs in agonadal women in the absence of estrogen stimulation, or after prolonged hormonal replacement with combination of estrogen and progesterone replacement in which menstrual sloughing does not take place. It is tempting to speculate that prolonged continuous progesterone stimulation of the endometrium produces a profound downregulation of estrogen receptors and that these may require priming prior to attaining an appropriate response.

To achieve luteinization of the endometrium, progesterone is started either on the day of oocyte retrieval or one day prior. Progesterone may be administered by the intramuscular or vaginal route, but progesterone delivery modes are more complex than those of estradiol. The reasons for

this are (i) much larger quantities of progesterone are necessary to achieve luteinization in the endometrium and (ii) progesterone is susceptible to metabolism by 5α-reductase in the skin, making transdermal delivery even more impractical due to the large size of the patch that would be required. In addition, oral progesterone is ineffective because it is rapidly metabolized. The advantages of the intramuscular route include higher serum progesterone levels and emotionally reassuring sense that the progesterone has been delivered to the body of the recipient. Many programs in the United States still utilize this mode of delivery with a dose of 50 to 100 mg daily. Even lower doses of 25 to 50 mg/day results in luteal phase serum levels of progesterone, a secretory endometrium, and good pregnancy rates (95). Supra-physiological doses of intramuscular progesterone do not appear to have an adverse effect on the maturation process (83), but doses lower than the standard result may have a negative impact (96).

Vaginal progesterone has generally been formulated in three ways: capsules containing micronized progesterone powder, a silastic ring that gradually releases progesterone, and a cream formulation. When compared with intramuscular administration of progesterone, vaginal micronized progesterone results in lower serum levels, but higher endometrial levels of progesterone. Its administration results in similar histological findings (97,98). For this reason, we have all but abandoned the intramuscular route, reserving it only for those patients who dislike the use of vaginal suppositories. Results from Europe on the use of a vaginal ring releasing 10 to 20 nmol/L of progesterone for 90 days have been favorable (99). In the United States, Crinone®, formulated as a cream, and administered vaginally twice daily, has been shown to result in in-phase biopsies (100) and pregnancy rates similar to those achieved with intramuscular progesterone (101).

EMBRYO TRANSFER

Embryo transfer is performed in the same manner as in conventional IVF. Pregnancies have been reported after transfer of embryos at any stage in development and after zygote intrafallopian transfer and tubal embryo transfer. In general, the recipient starts progesterone the day of or day prior to oocyte retrieval (102,103). As with conventional IVF cycles, the use of transabdominal ultrasound guidance during transcervical embryo transfer appears to improve the pregnancy and implantation rates (104).

Embryos derived from oocytes donated by young oocyte donors have very good implantation potential. Therefore, it is reasonable to limit the number of embryos that are transferred at one time. Recent studies have reported that the transfer of two versus three embryos results in similar pregnancy and implantation rates and a lower triplet and multiple rate

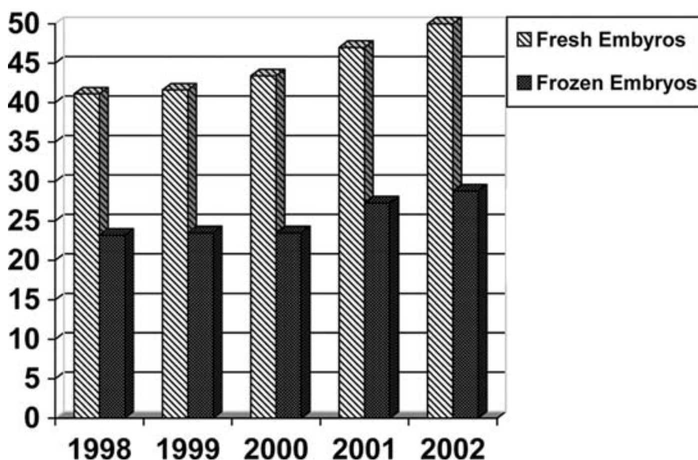

Figure 1 Percentage of transfers resulting in live births after oocyte donation by year. *Source*: From Ref. 107.

(14,105). Transfer of one embryo resulted in a slight decrease in pregnancy rate, but the probability of twins was greatly reduced (106).

PREGNANCY OUTCOMES

Live birth rates after oocyte donation have risen consistently, with pregnancy rates from fresh embryos being higher than those from cryopreserved ones (Fig. 1) (107). Pregnancy loss after visualization of fetal heart motion has been reported to occur in 5.7% of singleton pregnancies after oocyte donation (108). In the setting of multiple gestation, the probability of absorption of one or more embryos is 19% to 28% (108,109). The risk of fetal loss appears to correlate with the number of gestational sacs. Although patients with multiple gestations are more likely to have bleeding, their miscarriage rates (loss of all gestational sacs) does not appear to be increased (109). Most losses occur prior to the ninth week; thus selective reduction is generally delayed until after this time.

Pregnancies after oocyte donation appear to be associated with higher rates of pregnancy-induced hypertension, when recipients are compared to age-matched controls (110,111) (Table 2). It is not clear whether the increased incidence of pre-eclampsia is due to underlying disease or secondary to the oocyte donation itself. Serhal and Craft proposed that it may be due to inadequate immunoprotection of the fetoplacental unit (116). As is the case with all assisted reproduction, oocyte donation is associated with higher rates of multiple gestation, but pre-term delivery rates (at least for singleton gestations) do not appear to be significantly higher than those of the general population. Deliveries of multiples and even singletons most commonly occur via cesarean section (Table 2).

Table 2 Antenatal and Delivery Complications in Pregnancies After Oocyte Donation

Study (Reference)	Pregnancy-induced hypertension (%)	Pre-term delivery (%)	Cesarean section (%)
Singletons			
Sheffer-Mimouni et al. (112) ($n = 134$)	22.6	14.9	72
Abdalla et al. (113) ($n = 105$)	21	13	—
Soderstrom-Anttila et al. (114) ($n = 39$)	29	13	51
All Pregnancies			
Soderstrom-Anttila et al. (114) ($n = 51$)	31	30	57
Pados et al. (115) ($n = 52$)	32.7	1.9	63.5

INFANT OUTCOMES

Perinatal mortality and neonatal malformation rates do not appear to differ significantly from the general population. However, infants conceived after oocyte donation are at higher risk for low birth weight and small for gestational age (Table 3). In a study of children up to five years of age conceived with oocyte donation, all were healthy. Growth and development was similar to children in the general population (118). Parenting after oocyte donation is associated with more positive parent–child relationships and greater emotional involvement with the child compared to parenting after natural conception (119).

ADVANCED REPRODUCTIVE AGE

Prior to 1990, no series had specifically addressed the issue of oocyte donation to women of advanced reproductive age. Oocyte donation was primarily conceived of as a therapy for young women with premature ovarian failure, rather than as a means of overcoming the age-related decline in fertility. It was a serendipitous finding, therefore, that the high success rates with oocyte donation observed in the younger age group were mirrored in the women over 40 (22). The concept of offering fertility therapy to women over the age of 40 was not novel; rather, it was the observation that the success rates were not influenced by the age of the recipient that was remarkable. Several reports confirmed the efficacy of oocyte donation in the older group (120–122), and subsequently, it was demonstrated that pregnancies could be achieved in women over the age of 50 (30,123).

Table 3 Neonatal Complications in Singleton Pregnancies After Oocyte Donation

Study (Reference)	Perinatal mortality (%)	Neonatal malformations (%)	Low birth weight (%)	Small for gestational age (%)
Sheffer-Mimouni et al. (112) ($n = 134$)	0	2.2	14.9	7.6
Abdalla et al. (113) ($n = 105$)	—	—	18	15
Soderstrom-Anttila et al. (114) ($n = 51$)	3.3	5.1	10	5
Pados et al. (115) ($n = 52$)	1.7	0	—	—
Remohi et al. (117) ($n = 188$)	2.7	1	—	—

Pre-cycle screening of women of advanced reproductive age is extensive. As routine health maintenance in the older population includes additional testing, it is reasonable to require such testing prior to initiating therapy (Table 4). All recipients undergo a practice hormone replacement cycle of estrogen and progesterone with an endometrial biopsy on the seventh day of progesterone administration. With estrogen dosing adjusted to yield a normal mid-luteal endometrial histology, implantation and pregnancy rates comparable to younger women can be obtained (32).

One of the concerns about this type of therapy was the issue of obstetrical complications that might arise as a result of the advanced age of the new mothers. In a study of 77 postmenopausal women $\geq$50 years, we found that pregnancy rates, multiple gestation rates, and spontaneous abortion rates were similar to those of younger recipients (124). The incidence of gestational diabetes, pre-eclampsia, and cesarean section appears to be increased in this older age group (Table 5).

Although there does not appear to be any definitive medical reason for excluding these women from attempting pregnancy, provision of assisted reproductive technologies to postmenopausal women has remained controversial. It has been argued that oocyte donation to postmenopausal females may result in greater gender equality and reproductive freedom (129). However, arguments against it have focused on the impact to the children. Concerns have been raised about the physical and parenting capabilities

Table 4 Screening Tests for Prospective Recipients of Oocyte Donation Over the Age of 45 Years

Medical
Complete blood count
Blood chemistry panel
Thyroid-stimulating hormone
Fasting cholesterol panel
Glucose tolerance test
Coagulation parameters
Urinalysis
Papanicolaou test
Mammogram
Chest roentgenogram
Treadmill and baseline electrocardiogram
Colonoscopy (if over 50)
Reproductive
Transvaginal ultrasound
Endometrial biopsy and practice embryo transfer (day 21 of practice cycle)
Hysterosalpingogram or saline injection sonography
Infectious disease screen
HIV
HTLV I/II
VDRL
HbsAg
Hep C Ab
Preconceptual and psychosocial counseling

Abbreviations: HTLV, human T-cell lymphotropic virus; HbsAG, hepatitis B surface antigen; VDRL, veneral disease research laboratory.

Table 5 Obstetrical Outcomes After Oocyte Donation to Women of Advanced Reproductive Age

Study (Reference)	Age of subjects	Pregnancy-induced hypertension (%)	Gestational diabetes mellitus (%)	Cesarean section (%)
Paulson et al. (124) ($n = 77$)	≥50	35	20	78
Borini et al. (125) ($n = 34$)	≥50	22	11	—
Sauer et al. (126) ($n = 162$)	≥45	10.8	8	64.8
Antinori et al. (127) ($n = 1150$)	≥45	11.8	0.8	75
Porreco et al. (128) ($n = 50$)	≥45	42	8	64

of these older parents. To date, there have been no studies showing any adverse impact, thus concerns are only theoretical at this time point.

SUMMARY

Oocyte donation is a logical extension of the technology of IVF, designed to overcome blocks to fertility caused by oocyte problems. The details of an oocyte donation cycle are very similar to those of the standard IVF. Stimulation of the donor is similar to the standard IVF, yet may be simpler, because poor responder protocols are rarely, if ever, needed and concerns about hyperstimulation are at least partially mitigated by the fact that donors do not become pregnant. Preparation of the recipient endometrium and synchronization between donor and recipient are also achieved with relative ease, although it is possible that a superior approach to both may be discovered in the future. Donors as well as recipients should be medically screened and offered psychosocial counseling to address issues of third-party parenting. As oocyte donation bypasses all oocyte problems, it has increasingly been applied to the age-related block to conception in women of advanced reproductive age. It is possible to establish pregnancies in menopausal women over 50 years of age, although this practice is somewhat controversial. Obstetric outcomes after oocyte donation appear similar to those after other assisted reproductive technologies, with a possible increase in the incidence of hypertensive disorders of pregnancy.

REFERENCES

1. Buster JE, Bustillo M, Thorneycroft IH, et al. Non-surgical transfer of in vivo fertilised donated ova to five infertile women: report of two pregnancies. Lancet 1983; 2:223.
2. Bustillo M, Buster JE, Cohen SW, et al. Delivery of a healthy infant following nonsurgical ovum transfer. JAMA 1984; 251:889.
3. Bustillo M, Buster JE, Cohen SW, et al. Nonsurgical ovum transfer as a treatment on infertile women. preliminary experience. JAMA 1984; 251:1171.
4. Sauer MV, Anderson RE, Paulson RJ. A trial of superovulation in ovum donors undergoing uterine lavage. Fertil Steril 1989; 51:131.
5. Trounson A, Leeton J, Besanko M, et al. Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. Br Med J 1983; 286:835.
6. Lutjen P, Trounson A, Leeton J, Findlay J, et al. The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. Nature 1984; 307:174.
7. Devroey P, Smitz J, Camus M, Wisanto A, et al. Synchronization of donor's and recipient's cycles with GnRH analogues in an oocyte donation programme. Hum Reprod 1989; 4:270.

8. Sauer MV, Paulson RJ, Macaso TM, Francis-Hernandez M, et al. Establishment of a nonanonymous donor oocyte program: preliminary experience at the University of Southern California. Fertil Steril 1989; 52:433.
9. Sauer MV, Stein AL, Paulson RJ, Moyer DL. Endometrial responses to various hormone replacement regimens in ovarian failure patients preparing for embryo donation. Int J Gynecol Obstet 1991; 35:61.
10. Kavic SM, Sauer MV. Oocyte donation treats infertility in survivors of malignancies: ten-year experience. J Assist Reprod Genet 2001; 18:181.
11. Hovatta O, Soderstrom-Anttila V, Foudila T, Tuomivaara L, et al. Pregnancies after oocyte donation in women with ovarian failure caused by an inactivating mutation in the follicle stimulating hormone receptor. Hum Reprod 2002; 17:124.
12. Karnis MF, Zimon AE, Lalwani SI, Timmreck LS, et al. Risk of death in pregnancy achieved through oocyte donation in patients with Turner syndrome: A National Survey. Fertil Steril 2003; 80:498.
13. Shulman A, Frenkel Y, Dor J, Levran D, et al. The best donor. Hum Reprod 1999; 14:2493.
14. Mirkin S, Gimeno TG, Bovea C, Stadtmauer L, et al. Factors associated with an optimal pregnancy outcome in an oocyte donation program. J Assist Reprod Genet 2003; 20:400.
15. Harris SE, Faddy M, Levett S, Sharma V, et al. Analysis of donor heterogeneity as a factor affecting the clinical outcome of oocyte donation. Hum Fertil 2002; 5:193.
16. Abdalla HI, Baber R, Kirkland A, Leonard T, et al. A report on 100 cycles of oocyte donation; factors affecting the outcome. Hum Reprod 1990; 5:1018.
17. Wong IL, Legro RS, Lindheim SR, Paulson RJ, et al. Efficacy of oocytes donated by older women in an oocyte donation programme. Hum Reprod 1996; 11:820.
18. Stolwijk AM, Zielhuis GA, Sauer MV, Hamilton CJCM, et al. The impact of the woman's age on the success of standard and donor in vitro fertilization. Fertil Steril 1997; 67:702.
19. Faber BM, Mercan R, Hamacher P, Muasher SJ, et al. The impact of an egg donor's age and her prior fertility on recipient pregnancy outcome. Fertil Steril 1997; 68;370.
20. Balmaceda JP, Bernardini L, Ciuffardi I, Felix C, et al. Oocyte donation in humans: a model to study the effect of age on embryo implantation rate. Hum Reprod 1994; 9:2160.
21. Cohen MA, Lindheim SR, Sauer MV. Donor age is paramount to success in oocyte donation. Hum Reprod 2005; 14:2755.
22. Sauer MV, Paulson RJ, Lobo RA. A preliminary report on oocyte donation extending reproductive potential to women over 40. N Engl J Med 1990; 323:1157.
23. Navot D, Bergh PA, Williams MA, Garrisi J, et al. Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. Lancet 1991; 337:1375.
24. Flamigni C, Borini A, Violini F, Bianchi L, et al. Oocyte donation: comparison between recipients from different age groups. Hum Reprod 1993; 8:2088.

25. Cano F, Simon C, Remohi I, Pellicer A. Effect of aging on the female reproductive system: evidence for a role of uterine senescence in the decline of female fecundity. Fertil Steril 1995; 64:584.
26. Abdalla HI, Wren ME, Thomas A, Korea L. Age of the uterus does not affect pregnancy or implantation rates; a study of egg donation in women of different ages sharing oocytes from the same donor. Hum Reprod 1997; 12:827.
27. Garcia-Velasco JA, Isaza V, Caligara C, Pellicer A, et al. Factors that determine discordant outcome from shared oocytes. Fertil Steril 2003; 80:54.
28. Guanes PP, Remohi J, Gallardo E, Valbuena D, et al. Age does not affect uterine resistance to vascular flow in patients undergoing oocyte donation. Fertil Steril 1996; 66:265.
29. Sauer MV, Miles RA, Dahmoush L, Paulson RJ, et al. Evaluating the effect of age on endometrial responsiveness to hormone replacement therapy: a histologic, ultrasonographic, and tissue receptor analysis. J Assist Reprod Genet 1993; 10:47.
30. Sauer MV, Paulson RJ, Lobo RA. Pregnancy after age 50: application of oocyte donation to women after natural menopause. Lancet 1993; 341:321.
31. Paulson RJ, Hatch IE, Lobo RA, Sauer MV. Cumulative conception and live birth rates after oocyte donation: implications regarding endometrial receptivity. Hum Reprod 1997; 12:835.
32. Toner JP, Grainger DA, Frazier LM. Clinical outcomes among recipients of donated eggs: an analysis of the U.S. national experience, 1996–1998. Fertil Steril 2002; 78:1038.
33. Moomjy M, Cholst I, Mangieri R, Rosenwaks Z. Oocyte donation: insights into implantation. Fertil Steril 1999; 71:15.
34. Eldar-Geva T, Meagher S, Healy DL, MacLachlan V, et al. Effect of intramural, subserosal, and submucosal uterine fibroids on the outcome of assisted reproductive technology treatment. Fertil Steril 1998; 70:687.
35. Mukherjee T, Copperman AB, McCaffrey C, Cook CA, et al. Hydrosalpinx fluid has embryotoxic effects on murine embryogenesis: a case for prophylactic salpingectomy. Fertil Steril 1996; 66:851.
36. Zenke U, Chetkowski RJ. Transfer and uterine factors are the major recipient-related determinants of success with donor eggs. Fertil Steril 2004; 82:850.
37. Wattanakumtornkul S, Damario MA, Stevens Hall SA, Thornhill AR, et al. Body mass index and uterine receptivity in the oocyte donation model. Fertil Steril 2003; 80:336.
38. Bellver J, Rossal LP, Bosch E, Zuniga A, et al. Obesity and the risk of spontaneous abortion after oocyte donation. Fertil Steril 2003; 79:1136.
39. Pellicer A, Navarro J, Bosch E, Garrido N, et al. Endometrial quality in infertile women with endometriosis. Ann NY Acad Sci 2001; 943:122.
40. Spandorfer SD, Moomjy M, Davis OK, Barmat LI, et al. Oocyte donation: does a previous attempt affect a subsequent attempt? Fertil Steril 1998; 70:222.
41. Opsahl MS, Blauer KL, Black SH, Dorfmann A, et al. Pregnancy rates in sequential in vitro fertilization cycles by oocyte donors. Obstet Gynecol 2001; 97:201.
42. Caligara C, Navarro J, Vargas G, Simon C, et al. The effect of repeated controlled ovarian stimulation in donors. Hum Reprod 2001; 16:2320.

43. Practice Committee of the American Society for Reproductive Medicine. Repetitive oocyte donation. Fertil Steril 2004; 82(suppl 1):S158.
44. The American Society for Reproductive Medicine. Guidelines for oocyte donation. Fertil Steril 2004; 82(suppl 1):S13.
45. Practice Committee of the Society for Assisted Reproductive Technology, Practice Committee of the American Society for Reproductive Medicine. American Society for Reproductive Medicine/Society for Assisted Reproductive Technology position statement on West Nile Virus. Fertil Steril 2005; 83:527.
46. The American Society for Reproductive Medicine. Psychological assessment of gamete donors and recipients. Fertil Steril 2004; 82(suppl 1):S18.
47. The American Society for Reproductive Medicine. Minimal genetic screening for gamete donors. Fertil Steril 2004; 82(suppl 1):S22.
48. Zenke U, Chetkowski RJ. Inclusion of heterozygotes for cystic fibrosis in the egg donor pool. Fertil Steril 2002; 78:557.
49. Lindheim SR, Sauer MV. Upper genital-tract screening with hysterosonography in patients receiving donated oocytes. Int J Gynecol Obstet 1998; 60:47.
50. Cohen MA, Lindheim SR, Sauer MV. Hydrosalpinges adversely affect implantation in donor oocyte cycles. Hum Reprod 1999; 14:1087.
51. Sauer MV, Paulson RJ. Pelvic abscess complicating transcervical embryo transfer. Am J Obstet Gynecol 1992; 166:148.
52. Surrey ES, Schoolcraft WB. Laparoscopic management of hydrosalpinges before in vitro fertilization–embryo transfer: salpingectomy versus proximal tubal occlusion. Fertil Steril 2001; 75:612.
53. USE Fertility Recipient Regimen, USC Fertility, 2005 (not published).
54. Sauer MV, Paulson RJ, Moyer DL. Assessing the importance of endometrial biopsy prior to oocyte donation. J Assist Reprod Genet 1997; 14:125.
55. Potter DA, Witz CA, Burns WN, Brzyski RG, et al. Endometrial biopsy during hormone replacement cycle in donor oocyte recipients before in vitro fertilization–embryo transfer. Fertil Steril 1998; 70:219.
56. Barash A, Dekel N, Fieldust S, Segal I, et al. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. Fertil Steril 2003; 79:1317.
57. Jun SH, Racowsky C, Fox JH, Hornstein MD. The role of preparatory cycles in ovum donation recipients: a retrospective study. J Assist Reprod Genet 2004; 21:377.
58. Shapiro H, Cowell C, Casper RF. The use of vaginal ultrasound for monitoring endometrial preparation in a donor oocyte program. Fertil Steril 1993; 59:1055.
59. Hofmann GE, Thie J, Scott RT Jr., Navot D. Endometrial thickness is predictive of histologic endometrial maturation in women undergoing hormone replacement for ovum donation. Fertil Steril 1996; 66:380.
60. Sauer MV, Paulson RJ, Lobo RA. Comparing the clinical utility of GnRH antagonist to GnRH agonist in an oocyte donation program. Gynecol Obstet Invest 1997; 43:215.
61. Al-Inany H, Aboulghar M. Gonadotropin-releasing hormone antagonists for assisted conception (Cochrane Review). Cochrane Database Syst Rev 2001; 4:CD001750.

62. Thong KJ, Yong PY, Menezes Q. The administration of the GnRH antagonist, cetrorelix, to ooctye donors simplifies oocyte donation. Hum Reprod 2003; 18:1256.
63. Lindheim SR, Morales AJ. GnRH antagonists followed by a decline in serum estradiol results in adverse outcomes in donor oocyte cycles. Hum Reprod 2003; 18:2048.
64. Acevedo B, Sanchez M, Gomez JL, Cuadros J, et al. Luteinizing hormone supplementation increases pregnancy rates in gonadotropin-releasing hormone antagonist donor cycles. Fertil Steril 2004; 82:343.
65. Tesarik J, Mendoza C. Effects of exogenous LH administration during ovarian stimulation of pituitary down-regulated young oocyte donors on oocyte yield and developmental competence. [see comment]. Hum Reprod 2002; 17:3129.
66. Wong IL, Morris RS, Lobo RA, Paulson RJ, et al. Isolated polycystic morphology in ovum donors predicts response to ovarian stimulation. Hum Reprod 1995; 10:524.
67. Kavic S, Cohen MA, Sauer MV, Lindheim SR. Controlled ovarian hyperstimulation. Relationship of early serum E2 levels to the ultimate response of oocyte donors. J Reprod Med 2001; 46:637.
68. Pena JE, Chang PL, Chan LK, Zeitoun K, et al. Supraphysiological estradiol levels do not affect oocyte and embryo quality in oocyte donation cycles. Hum Reprod 2002; 17:83.
69. Sauer MV, Paulson RJ. Mishaps and misfortunes: complications that occur in oocyte donation. Fertil Steril 1994; 61:963.
70. Sauer MV. Defining the incidence of serious complications experienced by oocyte donors: a review of 1000 cases. Am J Obstet Gynecol 2001; 184:277.
71. Sauer MV, Paulson RJ, Lobo RA. Rare occurrence of ovarian hyperstimulation syndrome in oocyte donors. Int J Gynecol Obstet 1996; 52:259.
72. Morris RS, Paulson RJ, Sauer MV, Lobo RA. Predictive value of serum oestradiol concentrations and oocyte number in severe ovarian hyperstimulation syndrome. Hum Reprod 1995; 10:811.
73. Morris RS, Paulson RJ. Ovarian hyperstimulation syndrome: classification and management. Contemp Obstet Gynecol 1994; 39:43.
74. Shoham Z, Weissman A, Barash A, Borenstein R, et al. Intravenous albumin for the prevention of severe ovarian hyperstimulation syndrome in an in vitro fertilization program: a prospective, randomized, placebo-controlled study. Fertil Steril 1994; 62:137.
75. Aboulghar MA, Mansour RT, Serour GI, Amin Y. Ultrasonically guided vaginal aspiration of ascites in the treatment of severe ovarian hyperstimulation syndrome. Fertil Steril 1990; 53:933.
76. Isaza V, Garcia-Velasco JA, Aragones M, Remohi J, et al. Oocyte and embryo quality after coasting: the experience from oocyte donation. Hum Reprod 2002; 17:1777.
77. Sauer MV. Laparoscopy after multiple follicle aspirations fails to demonstrate pathology in oocyte donors. J Assist Reprod Genet 1996; 13:450.
78. Remohi J, Gutierrez A, Vidal A, Tarin JJ, et al. The use of gonadotrophin-releasing hormone analogues in women receiving oocyte donation does not affect implantation rates. Hum Reprod 1994; 9:1761.

79. Lelaidier C, de Ziegler D, Gaetano J, Hazout A, et al. Controlled preparation of the endometrium with exogenous oestradiol and progesterone: a novel regimen not using a gonadotrophin-releasing hormone agonist. Hum Reprod 1992; 7:1353.
80. Navot D, Laufer N, Kopolovic J, Rabinowitz R, et al. Artificially induced endometrial cycles and establishment of pregnancies in the absence of ovaries. N Engl J Med 1986; 314:806.
81. Krasnow JS, Lessey BA, Naus G, Hall L-LH, et al. Comparison of transdermal versus oral estradiol on endometrial receptivity. Fertil Steril 1996; 65:332.
82. Li TC, Cooke ID, Warren MA, Goolamallee M, et al. Endometrial responses in artificial cycles: a prospective study comparing four different oestrogen dosages. Br J Obstet Gynaecol 1992; 99:751.
83. Navot D, Anderson TL, Droesch K, Scott RT, et al. Hormonal manipulation of endometrial maturation. J Clin Endocrinol Metab 1989; 68:801.
84. Borini A, Dal Prato L, Bianchi L, Violini F, et al. Effect of duration of estradiol replacement on the outcome of oocyte donation. J Assist Reprod Genet 2001; 18:185.
85. Tourgeman DE, Slater CC, Stanczyk FZ, Paulson RJ. Endocrine and clinical effects of micronized estradiol administered vaginally or orally. Fertil Steril 2001; 75:200.
86. Noyes N, Hampton BS, Berkeley A, Licciardi F, et al. Factors useful in predicting the success of oocyte donation: a 3-year retrospective analysis. Fertil Steril 2001; 76:92.
87. Abdalla HI, Brooks AA, Johnson MR, Kirkland A, et al. Endometrial thickness: a predictor of implantation in ovum recipients? Hum Reprod 1994; 9:363.
88. Remohi J, Ardiles G, Garcia-Velasco JA, Gaitan P, et al. Endometrial thickness and serum oestradiol concentrations as predictors of outcome in oocyte donation. Hum Reprod 1997; 12:2271.
89. Sundstrom P. Establishment of a successful pregnancy following in-vitro fertilization with an endometrial thickness of no more than 4 mm. Hum Reprod 1998; 13:1550.
90. Bustillo M, Krysa LW, Coulam CB. Uterine receptivity in an oocyte donation programme. Hum Reprod 1995; 10:442.
91. Check JH, Nowroozi K, Choe J, Lurie D, et al. The effect of endometrial thickness and echo pattern on in vitro fertilization outcome in donor oocyte–embryo transfer cycles. Fertil Steril 1993; 59:72.
92. Fanchin R, Righini C, Schönauer LM, Olivennes F, et al. Vaginal versus oral E_2 administration: effects on endometrial thickness, uterine perfusion, and contractility. Fertil Steril 2001; 76:994.
93. Weckstein LN, Jacobson A, Galen D, Hampton K, et al. Low-dose aspirin for ooctye donation in recipients with a thin endometrium: prospective, randomized study. Fertil Steril 1997; 68:927.
94. Letur-Konirsch H, Delanian S. Successful pregnancies after combined pentoxifylline–tocopherol treatment in women with premature ovarian failure who are resistant to hormone replacement therapy. Fertil Steril 2003; 79:439.
95. Tavaniotou A, Smitz J, Bourgain C, Devroey P. Comparison between different routes of progesterone administration as luteal phase support in infertility treatments. Hum Reprod Update 2000; 6:139.

96. Li TC, Ramsewak SS, Lenton EA, Cooke ID, et al. Endometrial responses in artificial cycles: a prospective, randomized study comparing three different progesterone dosages. Br J Obstet Gynaecol 1992; 99:319.
97. Miles RA, Paulson RJ, Lobo RA, Press MF, et al. Pharmacokinetics and endometrial tissue levels of progesterone after administration by intramuscular and vaginal routes: a comparative study. Fertil Steril 1994; 62:485.
98. Bourgain C, Devroey P, Van Waesberghe L, Smitz J, et al. Effects of natural progesterone on the morphology of the endometrium in patients with primary ovarian failure. Hum Reprod 1990; 5:537.
99. Zegers-Hochschild F, Balmaceda JP, Fabres C, Alam V, et al. Prospective randomized trial to evaluate the efficacy of a vaginal ring releasing progesterone for IVF and oocyte donation. Hum Reprod 2000; 15:2093.
100. Toner JP. Vaginal delivery of progesterone in donor oocyte therapy. Hum Reprod 2000; 15(suppl 1):166.
101. Gibbons WE, Toner JP, Hamacher P, Kolm P. Experience with a novel vaginal progesterone preparation in a donor oocyte program. Fertil Steril 1998; 69:96.
102. Navot D, Scott RT, Droesch K, Veeck LL, et al. The window of embryo transfer and the efficiency of human conception in vitro. Fertil Steril 1991; 55:114.
103. Prapas Y, Prapas N, Jones EE, Duleba AJ, et al. The window for embryo transfer in oocyte donation cycles depends on the duration of progesterone therapy. Hum Reprod 1998; 13:720.
104. Lindheim SR, Cohen MA, Sauer MV. Ultrasound guided embryo transfer significantly improves pregnancy rates in women undergoing oocyte donation. Int J Gynecol Obstet 1999; 66:281.
105. Licciardi F, Berkeley AS, Krey L, Grifo J, et al. A two- versus three-embryo transfer: the oocyte donation model. Fertil Steril 2001; 75:510.
106. Soderstrom-Anttila V, Vilska S, Makinen S, Foudila T, et al. Elective single embryo transfer yields good delivery rates in oocyte donation. Hum Reprod 2003; 18:1858.
107. Centers for Disease Control. Assisted Reproductive Technology: Home. www.cdc.gov/reproductivehealth/ART
108. Legro RS, Wong IL, Paulson RJ, Lobo RA, et al. Multiple implantation after oocyte donation: a frequent but inefficient event. Fertil Steril 1995; 63:849.
109. Rodriguez-Gonzalez M, Serra V, Garcia-Velasco JA, Pellicer A, et al. The "vanishing embryo" phenomenon in an oocyte donation programme. Hum Reprod 2002; 17:798.
110. Salha O, Sharma V, Dada T, Nugent D, et al. The influence of donated gametes on the incidence of hypertensive disorders of pregnancy. Hum Reprod 1999; 14:2268.
111. Yaron Y, Ochshorn Y, Amit A, Kogosowski A, et al. Oocyte donation in Israel: a study of 1001 initiated treatment cycles. Hum Reprod 1998; 13:1819.
112. Sheffer-Mimouni G, Mashiach S, Dor J, Levran D, et al. Factors influencing the obstetric and perinatal outcome after oocyte donation. Hum Reprod 2002; 17:2636.
113. Abdalla HI, Billett A, Kan AKS, Baig S, et al. Obstetric outcome in 232 ovum donation pregnancies. Br J Obstet Gynaecol 1998; 105:332.

114. Soderstrom-Anttila V, Tiitinen A, Foudila T, Hovatta O. Obstetric and perinatal outcome after oocyte donation: comparison with in-vitro fertilization pregnancies. Hum Reprod 1998; 13:483.
115. Pados G, Camus M, Van Steirteghem A, Bonduelle M, et al. The evolution and outcome of pregnancies from oocyte donation. Hum Reprod 1994; 9:538.
116. Serhal PF, Craft IL. Immune basis for pre-eclampsia: evidence from oocyte recipients. Lancet 1987; ii:744.
117. Remohi J, Gartner B, Gallardo E, Yalil S, et al. Pregnancy and birth rates after oocyte donation. Fertil Steril 1997; 67:717.
118. Soderstrom-Anttila V, Sajaniemi N, Tiitinen A, Hovatta O. Health and development of children born after oocyte donation compared with that of those born after in-vitro fertilization, and parents' attitudes regarding secrecy. Hum Reprod 1998; 13:2009.
119. Golombok S, Lycett E, MacCallum F, Jadva V, et al. Parenting infants conceived by gamete donation. J Fam Psychol 2004; 18:443.
120. Sauer MV, Paulson RJ, Lobo RA. Reversing the natural decline in human fertility: an extended clinical trial of oocyte donation to women of advanced reproductive age. JAMA 1992; 268:1275.
121. Pantos K, Meimeti-Damianaki T, Vaxevanoglou T, Kapetanakis E. Oocyte donation in menopausal women aged over 40 years. Hum Reprod 1993; 8:488.
122. Serhal PF, Craft IL. Oocyte donation in 61 patients. Lancet 1989; 1:1185.
123. Antinori S, Versaci C, Gholami GH, Panci C, et al. Oocyte donation in menopausal women. Hum Reprod 1993; 8:1487.
124. Paulson RJ, Boostanfar R, Saadat P, Mor E, et al. Pregnancy in the sixth decade of life: obstetric outcomes in women of advanced reproductive age. JAMA 2002; 288:2320.
125. Borini A, Bafaro G, Violini F, Bianchi L, et al. Pregnancies in post-menopausal women over 50 years old in an oocyte donation program. Fertil Steril 1995; 63:258.
126. Sauer MV, Paulson RJ, Lobo RA. Oocyte donation to women of advanced reproductive age: pregnancy results and obstetrical outcomes in patients 45 years and older. Hum Reprod 1996; 11:2540.
127. Antinori S, Gholami GH, Versaci C, Cerusico F, et al. Obstetric and prenatal outcome in menopausal women: a 12-year clinical study. Reprod Biomed Online 2003; 6:257.
128. Porreco RP, Harden L, Gambotto M, Shapiro H. Expectation of pregnancy outcome among mature women. Am J Obstet Gynecol 2005; 192:38.
129. The Ethics Committee of the American Society for Reproductive Medicine. Oocyte donation to postmenopausal women. Fertil Steril 2004; 82(suppl 1): S254.

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Uterine Receptivity

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INTRODUCTION

The aim of this chapter is to bring the reader up to date with current thinking about the role of uterine receptivity in in vitro fertilization (IVF). The contents have been substantially revised and updated since the last edition, including information on the wealth of new data currently emerging on global gene expression profiling of human endometrium.

Although some progress in understanding of human uterine receptivity has been made over the past 10 years, most workers in the field would support the view that there have not been any paradigm shifts in our understanding of this complex topic. The contribution that reduced uterine receptivity makes to human infertility remains unclear. Unfortunately, a number of issues prevent significant progress in understanding the role of uterine receptivity in human reproduction, including first and foremost, the ethical and practical constraints that limit mechanistic studies involving embryo implantation in the human. Without successful embryo implantation and pregnancy as an outcome measure, definitive studies investigating uterine receptivity remain problematic. Despite this, the ready availability of human endometrium from non-conception cycles under a wide range of normal and manipulated situations provides huge scope for investigating endometrial events that may characterize a "receptive state."

The use of IVF as a routine clinical treatment for infertility provides an important avenue for focusing basic and clinical research on the human reproductive processes from gametogenesis to fertilization and implantation. In particular, many of the research-oriented IVF programs are now focusing attention on the role of the non-receptive uterus in reducing embryo implantation rates following controlled ovarian hyperstimulation (COH). There has been considerable speculation that implantation failure due to reduced endometrial receptivity is one of the major remaining impediments to higher IVF pregnancy rates.

ANIMAL STUDIES AND CURRENT CONCEPTS ON UTERINE RECEPTIVITY

Although significant progress has been made over the past 10 years in understanding the basic mechanisms that control embryo implantation in a number of mammalian species and in particular in the mouse (1), interpreting these data in the context of human endometrial receptivity is difficult because of the significant differences in mechanisms of implantation between species (2).

It is important from the outset to appreciate that fundamental differences exist in how a receptive endometrium is achieved between the human and commonly studied species, such as the mouse and rat. In human females, an average non-conception reproductive cycle is approximately 28 days, typically comprising four days of menstruation, 10 days of ovarian follicular growth with rising circulating estrogen levels, and 14 days post-ovulation with elevated progesterone levels. In a conception cycle, implantation commences about 5–6 days post-ovulation, with the endometrium has been exposed to 10 days of rising estrogen followed by 5–6 days of circulating progesterone. There is no evidence yet to suggest that a receptive endometrium in the human can be achieved by anything other than estrogen exposure followed progesterone. In contrast, mice and rats have a 4-day reproductive cycle, and it has been shown that the uterus becomes receptive to the implanting blastocyst for a short period of time, a few days after the commencement of continuous progesterone administration (3,4). Priming estrogen prior to this progesterone as occurs in the human is not essential, although a small amount of "nidatory" estrogen must be given at some time after the commencement of progesterone for an implantation-receptive uterus to be established. The length of time that the endometrium remains receptive can be manipulated by altering the nidatory estrogen dose, with higher doses resulting in shorter receptive periods (5). Nidatory estrogen is not a feature of the human implantation process. In the mouse, implantation occurs five days after ovulation, with stimulation of the uterine cervix during copulation inducing a state of "pseudopregnancy" during which normal 4-day ovarian cyclicity is halted for long enough to allow implantation to occur.

Bearing in mind the major differences that have been found between the different species studied, a number of general concepts have arisen from animal studies which form the basis of our understanding of endometrial receptivity in the human. First, it is generally believed that in the presence of a healthy blastocyst, it is the uterus, appropriately conditioned by ovarian hormones, that predominantly determines the success or otherwise of implantation (6). The "implantation window" is defined as the period of time when the uterus is receptive to the implanting blastocyst. In addition to the receptive state, there is evidence in some species that the uterus goes through "implantation neutral" and "implantation hostile" states (7). During the neutral phase, the embryo can survive in the uterus but will not implant, whereas during the hostile phase the embryo is actively destroyed. How the uterus affects these receptive, neutral, and hostile states remains mostly unclear, although embryo toxic compounds have been demonstrated in uterine flushing from rats and mice taken at times other than when implantation normally occurs (7,8) and there is a suggestion that similar embryo toxic compounds occur in the human (9). Another study has suggested a major role for the uterine epithelium in preventing implantation unless the correct hormonal priming had occurred (10). In this work, the embryo was only able to implant in the unprimed mouse uterus once the epithelium had been removed.

Further support for the hypothesis that the uterus is the major controlling partner in the implantation process comes from numerous studies, demonstrating that mammalian embryos can initiate implantation-type vascular reactions in non-uterine tissues with a high degree of success regardless of the sex or hormonal status of the host. Ectopic sites that have been studied in this way include the anterior chamber of the eye (11,12), the kidney (13), the testis (14), and the spleen (15). Although tissues from ectopic sites respond differently to the implanting embryo from those in uterine sites and despite the fact that subsequent embryo development is often disorganized, the study of ectopic implantation can provide valuable insights into normal intra-uterine implantation (12,16). Indeed, normal development to term has been reported in the human following ectopic implantation (17).

A number of mammalian species (including some of the marsupials) utilize embryonic diapause or delayed implantation to maximize reproductive efficiency (18). During diapause, blastocyst development has arrested by the uterus until environmental or physiological conditions are suitable for pregnancy to continue. That control over diapause is entirely a uterine phenomenon and can easily be shown by removing the blastocyst from the uterine environment, while normal development was re-commence (19). Conversely, studies in cattle have shown that under certain conditions the uterus can induce the preimplantation embryo to accelerate its development in order to "catch up" the uterus and be ready to implant at the optimal time in terms of uterine receptivity (20).

The term "implantation window" can be misleading when it is used to imply that there is a single critical window in time that determines whether implantation will be successful or not. In its broadest sense, implantation is a continuum of increasingly complex events wherein two independent tissues fuse and pregnancy is established. A key concept in understanding this process is developmental "synchrony," defined as when the early embryo and the uterus are both developing at a rate such that they will be ready to commence and successfully continue implantation at the same time. Using the concept of developmental synchrony, the length of the implantation window can be defined as the amount of embryo–uterine developmental asynchrony that can be tolerated while still allowing successful implantation.

If the implantation process is defined as comprising a continuum of events that must remain synchronized between the endometrium and the embryo, then it is apparent that many different individual events, or combinations of events, may dictate whether the implantation process is successful. One example of a critical endometrial event that determines the success of implantation in the mouse is the expression of leukemia inhibitory factor (LIF) by the endometrial glands just before the onset of implantation (21). There are other critical events in the implantation process and undoubtedly many more that remain to be discovered. Identifying what these are, when they occur, and if they play limiting roles in human IVF remains a challenging goal.

THEORETICAL CALCULATIONS OF HUMAN UTERINE RECEPTIVITY

The Natural Cycle

Estimates of maximum human fecundability (defined as a percentage of women who will produce a full-term infant per menstrual cycle during which frequent intercourse occurs) range from 14.4% to 31.8% for various populations and age groups of women (22). Under normal circumstances a number of factors may contribute to this low success rate, including anovulation, intercourse at the wrong time, reduced semen quality, failure of egg or sperm transport, fertilization failure, embryonic mortality, and lack of uterine receptivity (23). Of these potential factors, it is probable that embryonic mortality contributes significantly (24) as a result of both chromosomal (25) and other developmental abnormalities within the embryo. This work suggests that in the natural conception cycle in humans, uterine factors may not play a major role in implantation failure.

Uterine Receptivity and Embryo Viability During Stimulated IVF Cycles

The large amount of human implantation data produced following superovulation and multiple embryo transfer by various IVF groups has provided

an avenue for exploring the relative contributions of embryonic and uterine factors to implantation failure in stimulated cycles (26–30). Statistical modeling of IVF data was first proposed by Speirs et al. (31) based on a hypothetical two-parameter model for implantation. This model assumed that each embryo had a probability (E) of survival and that in any given patient there was a probability (U) that the uterus would be receptive. Thus, when a single embryo was transferred the probability of pregnancy was represented by the product $U \times E$. This simplistic model made a number of assumptions: that U and E were independent, that U and E were the only factors influencing implantation, that one embryo successfully implanting did not influence the chance of other embryos implanting, and that the probabilities U and E did not alter significantly within each IVF group with time.

Results of an early analysis using maximum likelihood methods to derive values for U and E are presented in Table 1. Although these data are from COH protocols that are no longer in general use, they are still of value in that they provide numerical estimates of the relative contributions of the embryo versus the uterus in governing successful implantation, and clearly indicate that uterine receptivity plays a major role. A second, equally important finding was that these data provided the first evidence that different COH protocols might influence uterine receptivity, with protocols that used the anti-estrogen clomiphene citrate having lower theoretical receptivity than those that used human menopausal gonadotrophin (hMG) alone.

There have been a limited number of attempts to use more sophisticated statistical modeling to derive estimates for E and U (28–30). Although these studies have the potential to significantly improve the understanding of the parameters that govern successful implantation in IVF cycles, each study has been limited by the relative lack of IVF data available for analysis. For example, in an analysis of 288 women with or without hydrosalpinx undergoing IVF it was concluded that hydrosalpinx had no discernable

Table 1 Estimates for Uterine Receptivity (U) and Embryo Viability (E) Based on Implantation Data from 5 In Vitro Fertilization Groups (± simultaneous 95% confidence limits)

IVF group	Uterine receptivity (U)	Embryo viability (E)
Monash, Melbourne	0.31 ± 0.07	0.32 ± 0.06
Royal Women's, Melbourne	0.42 ± 0.11	0.23 ± 0.05
Queen Elizabeth, Adelaide	0.42 ± 0.17	0.28 ± 0.10
Norfolk, Virginia	0.64 ± 0.22	0.21 ± 0.08
Bourne Hall, Cambridge	0.36	0.43

Abbreviation: IVF, in vitro fertilization.
Source: From Refs. 26 and 27.

effect on uterine receptivity, but the high variability in the data suggested that greater numbers of subjects were required (30). Multi-center trials coordinated between a number of IVF programs to collect standardized data in sufficient quantities for meaningful statistical analysis would overcome this problem and it is perhaps surprising that in the current era of evidence-based medicine this has not yet happened.

The Human Implantation Window

There is good evidence that in the normal cycle, implantation occurs over a period of time (31). The first detection of urinary chorionic gonadotrophin in women attempting to conceive occurred over a range 6–18 days post-ovulation. However, among pregnancies that survived for at least six weeks, the range was only 6–12 days. Eighty-four percent of successful pregnancies had initial detection of urinary chorionic gonadotrophin on days 8, 9, or 10 post-ovulation. These data support the concept that optimal implantation rates occur with embryo–uterine developmental asynchrony of one day or less. However, successful implantation can still occurs with asynchrony of up to three days, whereas the implantation process may be initiated, but fail to progress normally, with asynchrony of up to nine days.

Two IVF-related procedures, freezing and donation, provide an opportunity whereby developing embryos can be replaced into the uterus outside the normal obligatory uterus–embryo developmental synchrony, thus allowing investigation of the length of the human implantation window. In previous reviews of published pregnancies established in patients receiving donated oocytes for premature ovarian failure (32,33), it was estimated that successful implantation occurred when the embryo was anything from 39 hours in front to 48 hours behind the uterus. These calculations give an implantation window of at least 3 1/2 days. A summary of these two reviews and other data, representing more than 120 pregnancies, is given in Table 2. The calculations in Table 2 are based on the assumption that, as in animals (3,4), the commencement of progesterone in the human will initiate a "clock" that results in the uterus passing through a receptive phase. Uterine age is thus calculated as the time from when the uterus first received progesterone, and embryonic age is taken from the time of ovulation.

Of particular interest in Table 2 is the work of Formigli et al. (48). These workers used embryos lavaged from the uteri of donors five days post-ovulation and report pregnancies in cases where the recipient was anything from four days in front to three days behind the donor at the time of ovulation. However, there may be possible sources of error in the calculations of timing in this study. For example, ovulation was only assessed by daily ultrasound of the ovaries monitoring for disappearance of the follicle, and could therefore be up to 24 hours out. More importantly, it was not possible to exclude the possibility of spontaneous pregnancy in five out of

Table 2 Summary of Data on Embryonic–Uterine Synchrony Based on Patients with Ovarian Failure Receiving Hormone Replacement Therapy for Donor Oocyte and Embryo Transfers

Source	Pregnancies reported	Uterus ahead of embryo (hrs)	Uterus behind embryo (hrs)
Monash donor oocyte (34) (Fixed cyclical protocol)	11		18–39
Monash donor oocyte (35,36) (Variable length protocol)	40		20–36
Salat-Baroux et al. (37)	4		36
Feichtinger and Kemeter (38)	1		18
Asche et al. (39)	6		18
Rosenwaks (40)	8	0–48(?)	0–24
Junca et al. (41)	4	0(?)	
Devroey et al. (42,43)	11	0–12(?)	
Navot et al. (44)	2	4–28	
Serhal and Craft (45,46)	18	12	
Abdalla et al. (47)	8	12	
Formigli et al. (48) (Not all ovarian failure recipients)	8	0–96	0–72

Note: The synchrony calculations are based on the two assumptions that in the natural cycle: (a) significant progesterone secretion starts 12 hours before ovulation and (b) fertilization occurs six hours after ovulation. (For more detail on embryo–uterine developmental synchrony). Where it has not been possible from the published information to accurately estimate the timing of embryonic development relative to the point in time that the uterus first received progesterone, a (?) has been placed next to the calculated synchrony.
Source: From Ref. 33.

six patients with ovarian function. Accepting these limitations, this study suggests that the human implantation window may be up to seven days, twice that previously demonstrated by donor oocyte and embryo results.

In a study where embryo–uterine synchrony was systematically varied to explore the length of the implantation window (49), pregnancies were achieved over a six-day window that equated to implantation occurring anywhere from days 18/19 to 23/24. Numbers were not large enough in this study to determine whether differences in success rate existed within this time frame. It may also be possible to artificially widen the implantation window by manipulating the pre- and peri-implantation endocrine environment. In one study, it has been reported that ovarian stimulation with gonadotrophin-releasing hormone (GnRH) agonist/hMG compared with hMG alone appears to widen the implantation window (50). In this work,

the time of implantation was estimated by regression analysis of serial human chorionic gonadotropin (hCG) measurements taken between 7 and 16 days after embryo transfer.

There continue to appear in the literature case reports where for various reasons significant embryo–uterus developmental asynchrony existed at the time of embryo transfer, but where successful pregnancy ensued (51). Despite such reports, the overwhelming view has remained that, wherever possible, embryo and uterus should be as close to each other as possible in terms of developmental synchrony.

It is highly unlikely for ethical reasons that systematic investigation of the length of the human implantation window will ever be undertaken. If animal models are assumed to be informative for the human, then it is probable that the highest human pregnancy rates will be achieved within a limited window of embryo–uterus developmental asynchrony and that success rates will reduce as asynchrony increases. It is possible that in some cases of asynchronous transfer, pregnancy may be established despite the timing being less than optimal and that the overall success rate would be significantly reduced if all embryo transfers were conducted with the same asynchrony.

Knowledge of the length of the implantation window that gives maximal implantation rates in the human is of critical significance to studies aimed at identifying endometrial markers for uterine receptivity. If the time over which the uterus is receptive to the implanting embryo is not identified, it clearly will not be possible to correlate changing endocrine, biochemical, and morphological endometrial parameters with receptivity.

THE IMPACT OF COH ON THE ENDOCRINE REQUIREMENTS FOR UTERINE RECEPTIVITY

It has been appreciated for some time that the endocrine requirements for successful implantation in the human are not as tightly controlled as in species, such as laboratory rodents. Not only is the normal human menstrual cycle highly variable in length, but the normal limits for levels of circulating estrogen and progesterone vary widely (52). This variability, and the lack of any easy way of investigating whether any particular hormonal pattern is more appropriate for successful implantation, has resulted in a relatively limited understanding of the amounts or sequence of estrogen and progesterone necessary to prepare the human endometrium for implantation.

Subsequent to IVF becoming a routine clinical treatment, large amounts of accurate endocrine data have been generated on the circulating levels of estrogen and progesterone during stimulated and natural cycles. More importantly, following IVF treatment, receptive cycles are identified by establishment of pregnancy, thus allowing correlation of endocrine profiles with successful implantation. Unfortunately this has still not provided sufficient information for a good theoretical understanding of the endocrine

requirements for uterine receptivity to be achieved. Issues that contribute to this lack of understanding include the problem that implantation failure may occur for reasons other than lack of uterine receptivity and the high level of natural variability within the population in parameters that influence fecundity.

The lack of a good theoretical understanding of the endocrine requirements for uterine receptivity has a direct impact on the IVF industry, each time new COH protocols are developed that alter endocrine profiles during a cycle in which embryo transfer occurs. Rather than being able to rapidly develop new COH protocols that give optimal endometrial receptivity, it can take several years for consensus to evolve through trial-and-error by multiple IVF programs. Clearly there exists a need for more fundamental and IVF-related research into endometrial requirements for implantation.

Follicular Phase Estrogen and the Effects of COH

Animal and human donor oocyte studies continue to reinforce the central role that estrogen and progesterone play in preparing the endometrium for implantation. Thus, although a number of critical genes downstream from estrogen and progesterone have been identified, the fundamental challenge is to define the normal limits and interaction for circulating levels of these two hormones that will result in optimal endometrial receptivity. Available evidence suggests that neither the absolute levels of estrogen nor the time for which the uterus is exposed to estrogen are critical. Certainly, COH protocols with circulating estrogen levels many times higher than the natural cycle give excellent pregnancy results, as do variable-length estrogen replacement protocols used in donor oocyte programs.

However, within these apparently wide tolerance limits, there is also evidence from the use of GnRH antagonists over the past five years that some relatively subtle alterations to the endocrine profile may compromise successful implantation through effects on the endometrium. Following the report of a successful IVF pregnancy using third-generation GnRH antagonists (53), a major multicenter dose-finding study reported reduced implantation and pregnancy rates with increasing GnRH antagonist dosage of $\geq$0.5 mg/day (54). The subsequent finding that frozen embryos from the different treatment groups in this study implanted at similar rates led to the conclusion that the inhibitory effect of the higher GnRH antagonist doses was on the endometrium rather than the embryo (55). A subsequent study reported that when comparing 188 women receiving hMG/GnRH antagonist versus 85 receiving GnRH agonist down-regulation followed by HMG, those receiving the antagonist had significantly lower average of 17β-estradiol levels (1625 vs. 2082 pg/ml) (56). Pregnancy rates and children born per embryo replaced were slightly lower in antagonist versus agonist treatment groups, however, these differences were not significantly different.

Subsequent studies have confirmed the lack of difference in implantation rates between different COH protocols that included GnRH agonist and antagonist treatment arms (57,58). This raises the key question of what the precise endometrial mechanism responsible for reduced implantation with higher doses of GnRH antagonist actually is. In a commentary addressing this issue, it was suggested that reduced implantation rates with higher doses of GnRH antagonist may be linked to transient reductions in circulating estrogen levels when the GnRH antagonist is first administered (59). Several IVF groups have made statements supporting the finding that implantation and pregnancy rates with COH using low doses of GnRH antagonists are similar to those with the more traditional GnRH agonist COH protocols and suggesting that reduced implantation rates seen with higher GnRH antagonist doses are potentially due to perturbations in LH and estradiol levels rather than direct effects of the GnRH antagonist (60–62). Data supportive of this concept comes from a study of 111 women with either a fixed or flexible start time for GnRH antagonist depending on lead follicle diameter (63). These authors reported that increased levels of LH and estradiol, as measured by area under the curve between initiation of stimulation and commencement of antagonist, identified unsuccessful cycles. The same group had earlier reported that in study of 55 women stimulated with FSH/GnRH antagonist, endometrial maturation at oocyte retrieval was advanced on average by 2.5 days (64). Advancement correlated with increased luteinising hormone (LH) at initiation of stimulation and duration of recombinant follicle-stimulating hormone (FSH) stimulation. Pregnancies were not achieved if endometrial advancement was more than three days.

There has also been the suggestion that the anti-implantation effects of GnRH antagonists may be due to a direct effect of the antagonist on extra-pituitary GnRH receptors, thus acting as a cell cycle inhibitor in the endometrium by decreasing the synthesis of growth factors (65). However, there is some dispute as to whether at the doses being routinely used for IVF, GnRH antagonists have any intrinsic effect at the receptor level (61). Further evidence against a direct effect of GnRH antagonist on the endometrium comes from a study where antagonist administration was initiated either on day one of the COH cycle or day six (66). Implantation and pregnancy rates were similar in the two groups, despite the day 1 group receiving over double the exposure to GnRH antagonist that the day six group received. The day one group also had significantly lower LH and estradiol levels, which in this study also made no difference to final implantation or pregnancy rates.

It is not easy to provide a definitive summary about the effects of GnRH antagonists on uterine receptivity. The evidence suggests that at higher doses GnRH antagonists inhibit implantation through an endometrial mechanism, although the original data were not extensive enough to

be statistically significant, and the studies are unlikely to ever be repeated. From a theoretical point of view, the event most likely to interfere with endometrial receptivity is a transient decrease in circulating estrogen during the follicular phase of the menstrual cycle at the time when GnRH antagonist treatment commences. Some well-designed studies to test this theory may help to elucidate mechanisms that govern human endometrial receptivity.

Secretory Phase Progesterone and the Need for Luteal Support

During the normal ovarian cycle, progesterone is produced by the corpus luteum following ovulation. In a non-conception cycle, there is a subsequent fall in circulating levels of progesterone towards the end of the cycle as the corpus luteum regresses, leading to menstrual shedding of the functionalis layer of the endometrium. If conception occurs, βhCG produced by the embryo acts on the corpus luteum to maintain progesterone production resulting in continuation of the pregnancy. COH to produce multiple oocytes for IVF can significantly alter normal progesterone production. In particular, the long GnRH agonist down-regulation protocols reduce endogenous LH production for several days after agonist administration has ceased, leading to reduced circulating progesterone levels (67,68). Given the fundamental role of progesterone in both the development of a receptive secretory phase endometrium and the ongoing support of pregnancy, most IVF programs use some form of luteal support following COH.

There have been two recent major reviews of luteal support in assisted reproduction cycles (69,70) which have concluded that luteal support increases pregnancy rates. The first of these reviews (69) undertook a meta-analysis of 30 studies, with nearly all these using the long GnRH agonist stimulation protocol. Both intramuscular (IM) hCG and progesterone conferred a benefit, with the authors concluding that progesterone was preferable due to the increased risk of ovarian hyperstimulation syndrome (OHSS) with hCG. Progesterone given IM conferred more benefit than progesterone given orally or vaginally. The authors were less able to draw meaningful conclusions about the most beneficial timing, duration, dose, or formulation of progesterone due to the small size and varied objectives of individual studies. In the second review (70), results from 59 published randomized controlled studies were analyzed. Some of the conclusions from this review included: (i) luteal phase support with hCG provided significant benefit in terms of increased ongoing pregnancy rates and decreased miscarriage rates, but only in cycles where GnRH agonist was used as part of the COH; (ii) the odds of OHSS occurring increased 20-fold in cycles where hCG was used for luteal support; (iii) progesterone use gave a small but significant increase in pregnancy rates when studies with and without GnRH agonist were grouped together, but no effect was seen on miscarriage rates; (iv) no significant differences were found between progesterone and hCG

other than the odds of OHSS increased twofold with hCG; (v) there were increased ongoing pregnancy and live births with IM compared to vaginal progesterone, and a non-significant decrease in clinical pregnancy with oral compared to IM or vaginal progesterone.

Although the reviews summarized above provide clear evidence in favor of luteal support following COH for IVF, key questions remain unanswered. These include whether luteal support is required for all different COH regimens, what the optimal dose is, what the best route of administration is, whether the dose should differ with different routes of administration, and when in pregnancy support should be stopped. As it is not yet possible to directly measure uterine receptivity, all studies to date have relied on pregnancy as an outcome. The use of an indirect measure such as pregnancy means that factors other than reduced uterine receptivity will be contributing to pregnancy failure, making it difficult to develop luteal phase support protocols that maximize pregnancy outcomes.

The need for luteal support after use of GnRH antagonists, as opposed to agonists, in COH protocols requires further investigation. GnRH antagonists are typically only given for a few days with a rapid recovery of endogenous LH production once administration ceases. In a study of 41 subjects undergoing intra-uterine insemination, luteal progesterone levels, and the duration of the luteal phase were not altered by use of GnRH antagonist to control the LH surge (71). This study suggests that luteal support may not be required with GnRH antagonists, although clearly further studies will be required to confirm this.

The importance of the luteal phase defect (LPD) in infertility is questionable. LPD is defined as a defect of corpus luteum progesterone output either in amount or duration which results in inadequate stimulation of the endometrium for implantation of the blastocyst (72). Although some authors claim significant success from treating patients diagnosed as having LPD (73), others claim that the incidence of LPD among the fertile population is no different to that among infertile women (74). The observation by Wentz et al. (75) from a study of 54 biopsies taken inadvertently during conception cycles that the endometrium was on average dated significantly earlier than expected, clearly proves that delayed endometrial maturation to a degree consistent with the definition of LPD does not necessarily preclude implantation.

Fixed- and Variable-Length Hormone Replacement Cycles

The first donor oocyte recipient-steroid replacement regimens developed were based on the standardized 28-day menstrual cycle, with increasing levels of estrogen reaching 6 mg daily during the first half of the cycle and progesterone commencing mid-cycle (44,76,77). Satisfactory pregnancy rates were achieved using this type of hormone replacement protocol, although there were practical problems associated with the very short period

of time during the 28-day cycle that the recipient could be synchronized with a donated oocyte or embryo.

The discovery that variable-length constant estrogen dose replacement cycles were just as effective for establishing pregnancy as fixed-length-increasing estrogen dose ones revolutionized donor oocyte treatment (39,45). With variable-length estrogen replacement cycles progesterone is given to the patient from either the day before, or the day of, oocyte collection from the donor. Variable-length protocols have the advantage of allowing a much wider time-span in which the donor and the recipient can be synchronized. Early studies showed no difference in pregnancy rates per embryo transfer between the fixed and the variable-length protocols (35), however, more patients receive transfers with the variable-length protocol compared to the fixed one, resulting in more patients becoming pregnant.

The success of variable-length-constant estrogen dose replacement cycles for establishing pregnancies in women receiving donated oocytes provides important insights into just how flexible the uterine requirement for estrogen is while still allowing development of a receptive endometrium. As variable-length protocols became established, successful pregnancies were reported with simulated follicular phases of 10–24 days (35), 5–35 days (49), and even up to 100 days with estrogen replacement of 6 mg/day (78). Although receptivity may not be optimal under circumstances where estrogen mediated breakthrough bleeding is occurring (78), the fact that pregnancies can occur with such a wide time-range of estrogen pre-treatment suggests that estrogen does not act on the uterus by initiating a time-sensitive series of molecular and cellular events, as apparently occurs with progesterone. This observation, based on clinical experience, will be important in guiding fundamental studies of uterine receptivity in the human.

To date there have been no attempts to ascertain if less than five days of estrogen can prepare the endometrium for implantation. It has been shown in rhesus monkeys (79), and some other animal species (3), that follicular phase estrogen is not necessary for the development of uterine receptivity. However, reports that a short follicular phase of five days results in significantly increased early pregnancy wastage (49) may be an indication that this is approaching the physiological minimum for the human.

A prospective study of endometrial response to four different dosages of estrogen in women without ovarian function (80) has shown that 1 mg/day of estradiol valerate does not give an adequate secretory transformation when followed by five days of progesterone replacement. The response to 2 and 4 mg/day, as well as to a regimen that increased sequentially from 1 to 6 mg/day was satisfactory. Markers of secretory transformation included routine histological dating, sub- and supra-nuclear vacuoles in glandular epithelium, gland morphometry, and immunohistochemistry of the secretory phase protein D9B1. Interestingly, endometrial thickness was reported as similar in all four protocols. Although not mentioned by these authors, it

seems possible that the reduced secretory response following 1 mg/day estradiol valerate may be due to a failure to induce adequate numbers of progesterone receptors. Whether or not such a "sub-optimal" endometrium could support implantation has not been tested.

Studies in which oocytes from one donor are given to two recipients with only one of the recipients subsequently becoming pregnant provide a model from which recipient based factors that influence pregnancy success can be investigated. In a study of 134 donation cycles involving 116 recipients, factors that showed a statistical association with successful pregnancy were: reduced presence of uterine pathology, endometrium greater than 8 mm in thickness, fewer difficult embryo transfers, more good embryos transferred, and more prior pregnancies (81). Although uterine factors are clearly identified as being important in this study, there are too many other significant associations for any definitive conclusions to be drawn. Identification of structural and functional differences between endometria from women in similar groups may identify parameters that can eventually be used to measure uterine receptivity.

THE SEARCH FOR MARKERS OF UTERINE RECEPTIVITY

Considerable research effort continues to be focused on the search for one or more endometrial parameters that definitively indicate receptivity for implantation. An ability to readily identify receptive human endometrium will be of major clinical value in both infertility and contraceptive medicine. Although there are many well-documented endometrial changes around the time of implantation, obligatory markers for human uterine receptivity remain elusive. Given that the implantation process is a continuum of events, it is possible that no one definitive marker for uterine receptivity exists and that receptivity may be better defined by a combination of key markers that occur over a period of time. Significant progress has been made in establishing endometrial changes that occur in both normal and abnormal menstrual cycles during the peri-implantation period and from this information a picture is building up of features presumed to be desirable for receptivity.

Endometrial Histology and Morphology

The endometrium undergoes a well-established series of histological and ultrastructural changes under the influence of estrogen and progesterone during the menstrual cycle (82–84). However, numerous different studies continue to throw doubt on the functional importance of these morphological changes with respect to uterine receptivity for implantation (85–89). Semi-quantitative morphometric analysis of a single biopsy taken inadvertently from a conception cycle of a patient receiving hormone replacement therapy for premature ovarian failure revealed an appearance that could

not by any degree be classified as typical for a mid-secretory stage receptive endometrium (85). Despite this, implantation had clearly occurred. In contrast, detailed morphometric analysis of peri-implantation endometrium has failed to demonstrate any difference between fertile controls and infertile subjects (90). More recently, a study of pinopode formation and integrin expression in six different groups of women concluded that although pinopode formation and integrin expression are closely related to normal endometrial maturation, this is irrespective of whether the endometrium is in-phase or out-of-phase. Thus, the potential usefulness of these two features as markers for uterine receptivity is questionable (88). In another study, endometrial glandular volume in IVF patients receiving CC/hMG for ovarian stimulation was significantly reduced compared with the normal menstrual cycle (87), whereas patients receiving GnRH agonist "flare" stimulation (85) had elevated glandular area. Despite these differences, which probably reflect differing levels of estrogenic stimulation, successful implantation and pregnancy occurred with both types of endometrium. In yet another detailed study of potential endometrial markers of receptivity, it was found that appearance of three of the most cited markers of receptivity, namely pinopods β3 and α4 integrin, do not correlate, thus questioning their utility as markers (89). In summary, implantation can occur in an endometrium with morphological features that are significantly different from normal, and there is no conclusive evidence yet available to show that any particular structural defect correlates specifically with reduced uterine receptivity.

The human uterine epithelial surface plasma membrane undergoes characteristic changes during the menstrual cycle. In particular, long thin surface microvilli found during the proliferative phase of the cycle under the influence of estrogen are replaced by short irregular surface projections under the influence of progesterone in the luteal phase (91). Based on works in the rat and mouse, where similar luminal epithelial projections are pinocytotic, these projections have been called pinopods. However, recent studies suggest that the human equivalent to the rodent pinopod may not be pinocytotic and should more correctly be called a uterodome (92).

It has been assumed from animal studies that the pinopod transformation of the plasma membrane is obligatory for successful implantation. Human studies using scanning electron microscopy have also proposed that these short irregular surface projections, or uterodomes, are transient markers of uterine receptivity (93). However, although implantation requires both progesterone and nidatory estrogen in rodents, pinopods will develop following progesterone alone and are thus not obligatory markers of a receptive uterus (92). Experimental evidence to support uterodomes as specific markers of receptivity in the human is lacking, whereas there is evidence of implantation occurring in the absence of uterodomes (85). Without convincing evidence to the contrary, serious doubt remains regarding the obligatory requirement of pinopods for successful implantation in the human.

Tight junctions are fundamental ultrastructural features of epithelial cells playing an important role in joining adjacent cell walls closely together and thus maintaining the integrity of the epithelium. For implantation to occur in the human, the embryo must breach the epithelium, raising the possibility that in the receptive uterus mechanisms may exist to reduce the integrity of the epithelial barrier. Freeze-fracture studies (94) have shown that epithelial tight junctions undergo a significant decrease in area between days 13 and 23 of the menstrual cycle, as well as a reduction in geometrical complexity. These data support the hypothesis that sex steroid conditioning of the human uterus for implantation causes a reduction in epithelial integrity. In a related study (95), the surprising observation was made that, in 9 out of 11 biopsies from six Turner's syndrome patients, there were no tight junctions, whereas in the remaining two biopsies, tight junctions were greatly reduced. Two of these Turner's patients subsequently had successful pregnancies. The observation that an apparently compromised epithelium does not appear to interfere with implantation is consistent with the hypothesis that the uterine epithelium normally acts as a barrier to the embryo, only becoming receptive for implantation when appropriately conditioned with estrogen and progesterone.

The Effects of Ovarian Stimulation on Endometrial Morphology

There have been numerous publications dealing with endometrial histopathology following ovarian stimulation (87,96–105). A comparison of results from some of these papers presents a confusing picture, due in part to methodological differences between studies. Factors that can influence endometrial histology in this type of work include stimulation regimen, endocrine response, patient age and cause of infertility, timing of the endometrial biopsy, and the subjective nature of histopathological classification of the biopsy. A brief review of published information on endometrial response to ovarian stimulation is provided below.

In patients given complete citrate (CC)/hMG and biopsied 2–4 days after oocyte retrieval, 8 out of 15 had hypotrophic endometrium that was thought to be unsuitable for nidation (96), and 9 out of 24 still had proliferative endometrium (97). In contrast, biopsies taken during the proliferative phase 24–48 hours after the end of CC administration showed advanced secretory changes in 10 out of 19 cases (98). In another study, a variety of endometrial defects were identified two days after oocyte retrieval in 84% of patients receiving CC/hMG and in 52% of patients receiving hMG (99). The same study reported that normal endometrial histology was only found in women less than 36 years of age.

Subjective histopathology may not identify abnormalities or structural changes that can be detected using other methods. In a study (106) of 17 women (12 received CC/hMG and five received buserelin/hMG) biopsied

two days after oocyte retrieval, 15 had histopathological dating that agreed exactly with the chronological age of the endometrium. In contrast, both morphometric analysis and ultrasound imaging techniques demonstrated significant differences in endometrial structure between women receiving the two different stimulation protocols. Morphometric techniques have subsequently been used to confirm that CC results in reduced endometrial glandular volume compared with normal menstrual cycle controls (87).

A study of patients receiving hMG alone and biopsied 1–3 days after the LH surge found 11 out of 22 to have histologically advanced endometrium (100). Considerable stroma-gland developmental dyssynchrony has been reported by the late luteal stage of the cycle in women treated with hMG alone (104), with the glands on average up to three days behind stroma. It has been argued that pituitary downregulation with GnRH agonist followed by ovarian stimulation with hMG does not cause abnormal endometrial development (102), although this finding is at odds with a study where 18 patients receiving Buserelin/hMG and biopsied on day 21 of the cycle had an average delay in endometrial maturation of 2.6 days (101). In a detailed study of a range of endometrial variables (105), numerous differences were noted between controls and women stimulated with either hMG, GnRH agonist downregulation/hMG, or GnRH agonist downregulation/hMG/progesterone. A generalized finding was that women stimulated with hMG or GnRH agonist downregulation/hMG had a more advanced endometrium in the early luteal phase. More recently, it has been shown that women stimulated with GnRH agonist downregulation/hMG had an endometrial glandular volume very similar to controls in the early secretory stage of the cycle, whereas women stimulated by GnRH agonist boost followed by hMG had considerably higher endometrial gland volume (87).

Ultrasound and Doppler Imaging of the Endometrium and Uterine Blood Flow

There have been major technological advances in ultrasound and Doppler imaging over the past 10 years, with every probability that novel, non-invasive imaging techniques will continue to be developed. These imaging modalities have been used to investigate at least three different aspects of uterine appearance that may relate to uterine receptivity, namely endometrial echogenicity, uterine blood flow, and uterine contractions (107). A major advantage of using ultrasound to investigate uterine receptivity is that it is non-invasive. However, despite the fact that appearances that are associated with increased implantation have been identified, studies with contradictory findings are not uncommon and obligatory markers of receptivity have yet to be identified.

When ultrasound-guided follicle aspiration replaced laparoscopy as the method of choice for IVF, it became inevitable that ultrasound imaging

would also be used to assess the endometrium. Early studies demonstrated that ultrasound can be used to differentiate between the endometrial response to different COH regimens, with the different images reflecting morphological differences between the endometria (106). Other early studies reported that endometrial thickness and pattern on the day before oocyte retrieval may be an indicator of the likelihood of achieving pregnancy (108). This was supported in part by the finding that a multilayered echogenic pattern was predictive of pregnancy, but that endometrial thickness was not (109). The endometrium changes from a hypoechogenic appearance during the follicular phase to hyperechogenic during the secretory phase, with the increased echogenicity developing from the basal is upwards. In a detailed study of endometrial echogenicity on the day of hCG administration, it was shown that higher pregnancy and implantation rates occur if the endometrium is less echogenic in the late follicular phase (107,110). The exact morphological features that underlie increasing endometrial echogenicity as the cycle progresses are unclear, although the assumption is that if the endometrium is prematurely advanced, as evidenced by an early increase in echogenicity, then receptivity will be reduced. Although this and other studies have found that a central hyperechoic line between two hypoechoic layers are more common in conception cycles, these parameters may be of limited value in a clinical setting due to considerable overlap between conception and non-conception cycles (111,112).

Uterine artery pulsatility measured by color Doppler has also been investigated as a predictor of implantation, with early reports showing that an increased pulsatility index at the time of embryo transfer may be associated with a reduced implantation rate (113,114). Color Doppler sonography can also be used to measure endometrial–subendometrial blood flow. It has been reported that the pregnancy rate for patients with detectable endometrial and subendometrial blood flow prior to embryo transfer was 47.8%, whereas with subendometrial flow alone it was 29.7%, and with no detectable flow, it was only 7.5% (115). In agreement with this study, it has been shown using color and power Doppler on the day of embryo transfer that absence of sub- and intra-endometrial color signal decreased the chances of pregnancy by an odds ratio of approximately eightfold (116). Interestingly, it has been reported that following COH using GnRH agonist downregulation, endometrial–subendometrial blood flow is significantly reduced compared to the natural cycle, with the inference being that uterine receptivity is also reduced (117). In contrast to the above reports of a link between endometrial blood flow and subsequent conception, others have found that neither Doppler sonography of the spiral or uterine arteries, nor measurement of endometrial thickness or volume allowed reliable prediction of subsequent IVF outcome (118).

There is currently little understanding of the physiological link between increased endometrial and subendometrial blood flow at the time

of embryo transfer and increased uterine receptivity. Further research into the morphological and molecular correlates of these Doppler ultrasound findings may help to develop better, clinically useful, non-invasive screening tests for uterine receptivity.

Uterine junctional zone contractility is exaggerated in IVF compared to natural cycles, with the implication being that the embryo is less likely to be expelled from a quiescent uterus following embryo transfer (119). Given that uterine junctional zone contractility decreases during the cycle, it is possible that pregnancy rates could be improved by increasing the time between oocyte retrieval and embryo transfer to take advantage of the reduction in contractility that occurs over this time period.

Endometrial Gene Expression

The development of microarray gene expression profiling as a routine technique allows whole tissue or cell global gene expression profiles to be rapidly determined and correlated with different structural, functional, and clinical parameters (120). Recent advances in molecular biology and reproductive physiology have raised questions about the reliability of morphological criteria in determining some aspects of clinical or biological function. According to established criteria (82), an endometrial biopsy is considered abnormal when there is out-of-phase histological maturation of more than two days, based on the assessment of a relatively small number of structural features. It has recently been shown that these structural features may be much less temporally distinct and discriminating than originally thought (121). With microarray technology now readily available in many laboratories, a number of studies have already been published investigating global endometrial gene expression at different stages of the menstrual cycle, and new studies are appearing in the literature on a regular basis.

In a microarray study of endometrial curettings obtained from 43 normal cycling women, we found a strong relationship between the grouping of endometrial samples based on gene expression profiles and their histopathological cycle stages (122). We also demonstrated that discordant patterns of gene expression may help to select endometrial samples with subtle abnormalities not readily identified by routine histopathology. This study also reinforced the concept of the menstrual cycle as a continuum of changing gene expression, rather than a series of discrete stages. Thus, global endometrial gene expression alters continuously over the 3–4 days when implantation is most commonly believed to occur, making a precise molecular definition of uterine receptivity problematical. We found that major changes in endometrial gene expression occur around the time of implantation, with a number of gene clusters showing peaks of expression at this time. These clusters included previously known and novel genes associated with implantation-related biological processes, such as cell adhesion, cell growth

and differentiation, and signal transduction. Examples of implantation associated genes identified by this study include Trophinin, transforming growth factor alpha (TGFα), platelet-derived growth factor alpha (PDGFα), fibroblast growth factor 2 (FGF2), and placental growth factor (PlGF).

There have been four recent microarray publications designed to find genes involved in implantation. Two groups compared late proliferative stage with mid-secretory stage (123–126) and two compared early-secretory with mid-secretory stages. The number of differentially expressed genes reported from each of these studies ranged between 146 and 332. It is interesting to note, however, that even though there were significant similarities in the experimental setup between the four studies and they were performed on the same microarray platform, the results share only four genes in common. These are granulysin, secreted phosphoprotein-1, apolipoprotein-D, and dickkopf-1. This result raises a cautionary note about the need for well-designed microarray studies with statistically valid sample numbers and the necessity for results to be validated by alternative techniques.

Gene microarray has also been used to investigate endometrial gene expression changes induced by COH (127). This study found more than 200 genes showed differential expression of $\geq$3-fold when GnRH agonist downregulation at HCG + 7 was compared with natural cycle at LH + 7. These results confirm the widely held view that COH has significant effects on endometrium and opens the way for future work to develop COH protocols that cause minimal endometrial disruption.

An increasing number of individual genes have been identified from animal studies as having key roles in the implantation process. To date, none of these have progressed to being of established clinical use, although it remains probable that if progress is to be made in predicting or treating human infertility, it will involve genes first identified from animal studies. Although it is not possible within the context of this chapter to review all the published uterine receptivity work that has been undertaken in animals, a limited number of studies and genes are worthy of mention.

One of the earliest reported genes with an obligatory role in implantation was LIF which is required for successful implantation in mice (21). Subsequent studies have shown that in the mouse endometrial receptivity is under dual control, being regulated both by the onset of LIF expression by the endometrial glands, and the release from inhibition of LIF receptor function in the luminal epithelium (128). The role of LIF in human implantation is less clear, due mostly to the inability to undertake investigations that involve mechanistic studies.

The HOX genes are transcription factors that are expressed in the adult uterus. Hoxa (10) plays a role in regulating endometrial stromal cell responsiveness to progesterone, and is necessary for uterine receptivity and implantation in mice (129). Recent evidence also suggests a role for HOX genes in human implantation, where decreased implantation rates

have been reported in women with altered HOX expression, and HOX genes have been proposed as markers of endometrial receptivity (130).

A recent study has identified lysophosphatidic acid receptor (LPA_3) mediated signaling as having an influence on implantation in the mouse, in part at least through the prostaglandin biosynthesis pathway (131). Another recently identified molecule that is essential for establishment of pregnancy in mice is proprotein convertase 6 (PC6), an endoprotease that regulates protein function through posttranslational activation of precursor proteins. In vivo blocking of uterine production of PC6 protein in mice resulted in total inhibition of implantation (132). Studies in the human and rhesus monkey have shown an upregulation of endometrial PC6 during the phase of uterine receptivity and at implantation, suggestive of a similarly important role in these species. Other genes of known importance in implantation include the transforming growth factor beta superfamily including the activins, members of the gp130 family, such as interleukin-11 (IL-11), the colony-stimulating factors, IL-1, and IL-15 (133).

AGE-RELATED CHANGES IN UTERINE RECEPTIVITY

Natural fecundity begins to fall from age 30 to 35, with the decline becoming more noticeable after age 40 (134). This reduction in fertility with increasing age is clearly evident among IVF patients (135,136), with a report that the implantation rate falls by 2.77% per year after the age of 35 years (137). Another recent example to support this observation is a study that involved 1263 women aged 40 years and above, undertaking 2705 cycles of artificial reproductive technology (138). Cumulative pregnancy rates ranged from 28.4% in women commencing treatment at the age of 40 years, down to 0% for those commencing at the age of 46 years. However, these studies do not identify which components of the reproductive process are affected by aging. Oocyte donation programmes have provided an important avenue for investigating the relative contributions of uterine and embryonic factors to the age-related decline in human fertility (139). At the present time, a consensus opinion on this issue appears to be that although the uterus may become less receptive to implantation and the maintenance of pregnancy with increasing age, the primary effect is due to declining oocyte quality (140).

Several studies have failed to identify any decrease in uterine receptivity with increasing age. Among recipient pairs of differing ages receiving oocytes from the same donor, there was no difference in pregnancy rates with increasing age (141). Similarly, pregnancy rates of 29% in women over the age of 50 (142) and 32.7% in women aged 50–62 (143) have been reported using oocytes donated by younger women. Thus, it seems clear that the human uterus can support pregnancy well after the end of the normal reproductive lifespan. On a cautionary note, however, it is important to recognize that significant maternal morbidity is associated with

postmenopausal pregnancy. In the second study mentioned above (143), gestational diabetes was present in 16.6% of the patients that delivered, compared to 0% of donors and 0.35% of the normal population and moderate or severe pre-eclampsia affected 41.6% of the subjects, compared with 0% of donors and 7.3% of the normal population.

If reduced oocyte quality is the major factor with increasing age, then the age of the oocyte donor, rather than that of the recipient, should be the key issue in determining pregnancy outcome. However, it has been reported that the age of the recipient, but not the donor, significantly affects pregnancy rate, falling from 50% in the 25–29 years age group to 9.7% in the 45–49 years age group (144). In addition, the etiology of the infertility was important in determining whether or not a successful outcome occurred, with 50% of women with primary ovarian failure becoming pregnant compared to only 18% of patients with secondary ovarian failure. It was also found that the pregnancy rate was significantly higher when the oocytes were donated from women who had already had children compared to donors who had not had children. Other studies have also found no difference in pregnancy rates when oocyte donor ages were analyzed (145); however, it was found that oocytes donated by older women had more chance of resulting in spontaneous abortions than oocytes from younger women. In this same study, recipients who conceived were significantly younger than those who did not, supporting the concept of reducing uterine receptivity with increasing age.

There are conflicting reports in the literature as to whether increasing recipient age results in increased pregnancy wastage. In one study looking at recipients of different ages who had shared oocytes from the same donor, the pregnancy rate dropped from 43.7% in women less than 40 years old to 24.5% in women aged between 40 and 49 years (143). The subsequent abortion rate was similar in both groups. In contrast, it has been reported elsewhere that women over 40 years receiving donated oocytes have similar implantation rates as younger women, but significantly higher abortion rates (146). This study suggested that this effect may be linked to later production of placental estrogen and progesterone by the older subjects.

It is possible that small subject numbers and random chance have led to some of the conflicting results discussed above. Another explanation is that major differences in the etiologies of both donors and recipients have blurred the overall trends associated with ageing. For example, women recruited through association with IVF programs may have a long history of subfertility, including undiagnosed oocyte and endometrial problems. Women who are more fertile who have already achieved a successful pregnancy will tend to drop out of these groups with time, leaving a study group that may be unrepresentative of the normal population.

There is little basic scientific evidence available to indicate what, if anything, might cause endometrial receptivity reduction with increasing

age. Significant differences have been demonstrated in some reproductive criteria between women aged 21–25 years and those 37–45 years (147). In the older group ovulation occurred two days later than the younger group and the leading follicle diameter was nearly 3 mm less. As expected, FSH and LH levels were elevated in the older women and somewhat surprisingly it was also reported that endometrial thickness was increased in the older women. No differences were seen in estrogen or progesterone levels. Serum FSH levels are elevated and inhibin levels reduced in women over the age of 40 years, but this is not reflected by any obvious changes in endometrial morphology (148). No difference has been found in histology, ultrasound, or steroid receptor content of the endometrium between women in age groups ranging from 25 to 60 years (149), and there is no evidence for an increase in uterine vascular impedance as measured by Doppler ultrasound in older women (150).

SUMMARY AND CONCLUSIONS

In summary, although there is general agreement that uterine receptivity is a key player in determining the outcome of IVF treatment, there remains a significant gap in our knowledge of either markers or mechanisms that would help in overcoming this problem. Despite restrictions on the research approaches that can be used in studies of embryo implantation in humans, a number of avenues offer promise for future work. These include, but are not limited to, theoretical modeling studies using sophisticated statistical and bioinformatic techniques on very large multi-center IVF data sets, non-invasive imaging techniques of the uterus and endometrium, and identification of endometrial gene and protein expression profiles that predict IVF treatment outcome. Animal models will always remain the primary approach for mechanistic studies; however, differences in mechanisms of implantation between species dictates that a strong focus should always be retained on the human. The ultimate goal for researchers in the field of uterine receptivity should be to identify markers that are reliable and specific enough to be of clinical use in decision-making during the IVF treatment process.

REFERENCES

1. Dey SK, Lim H, Das SK, et al. Molecular cues to implantation. Endocr Rev 2004; 25:341–373.
2. Finn CA, Porter DG. Implantation of ova. In: Finn CA, ed. The Uterus, Handbooks in Reproductive Biology. Vol. 1. London: Elek Science, 1975:57–73.
3. Humphrey KW. Induction of implantation of blastocysts transferred to ovariectomized mice. J Endocrinol 1969; 44:299–305.
4. De Feo VJ. Temporal aspect of uterine sensitivity in the pseudopregnant or pregnant rat. Endocrinology 1963; 72:305–316.

5. Ma WG, Song H, Das SK, Paria SK, Dey SK. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. PNAS 2003; 100:2963–2968.
6. Sherman MI, Wudl LR. The implanting mouse blastocyst. Cell Surf Rev 1976; 1:81–125.
7. Psychoyos A, Casimiri V. Factors involved in uterine receptivity and refractoriness. Prog Reprod Biol 1980; 7:143–157.
8. Weitlauf HM. Factors in mouse uterine fluid that inhibit the incorporation of ^{3}H-uridine by blastocysts in vitro. J Reprod Fert 1978; 52:321–325.
9. Psychoyos A, Roche D, Gravanis A. Is cholic acid responsible for embryo-toxicity of the post-receptive uterine environment? Hum Reprod 1989; 4:832–834.
10. Cowell TP. Implantation and development of mouse eggs transferred to the uteri of non-progestational mice. J Reprod Fert 1969; 19:239–245.
11. Runner MN. Development of mouse eggs in the anterior chamber of the eye. Anat Rec 1947; 98:1–17.
12. Rogers PAW, Macpherson AM, Beaton LA. Vascular response in a non-uterine site to implantation-stage embryos in the rat and guinea-pig: in vivo and ultrastructural studies. Cell Tissue Res 1988; 254:217–224.
13. Fawcett DW. The development of mouse ova under the capsule of the kidney. Anat Rec 1950; 108:71–91.
14. Kirby DRS. The development of mouse blastocysts transplanted to the scrotal and cryptorchid testis. J Anat 1963; 97:119–130.
15. Kirby DRS. Development of the mouse blastocyst transplanted to the spleen. J Reprod Fert 1963; 5:1–12.
16. Macpherson AM, Rogers PAW, Beaton LA. Vascular response in a non-uterine site to implantation stage embryos following interspecies transfers between the rat, mouse and guinea-pig. Cell Tissue Res 1989; 258:417–423.
17. Ombelet W, Vandermerwe JV, Van Assche FA. Advanced extrauterine pregnancy: description of 38 cases with literature survey. Obstet Gynecol Surv 1988; 43:386–397.
18. Sandell M. The evolution of seasonal delayed implantation. Quart Rev Biol 1990; 65:23–42.
19. Lundkvist Ö, Nilsson BO. Endometrial ultrastructure in the early uterine response to blastocysts and artificial deciduogenic stimuli in rats. Cell Tissue Res 1982; 225:355–364.
20. Garrett JE, Geisert RD, Zavy MT, Morgan GL. Evidence for maternal regulation of early conceptus growth and development in beef cattle. J Reprod Fert 1988; 84:437–446.
21. Stewart CL, Kaspar P, Brunet LJ, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992; 359:76–79.
22. Leridon H. Human Fertility. Chicago: University of Chicago Press, 1977.
23. Wood JW. Fecundity and natural fertility in humans. In: Milligan SR, ed. Oxford Reviews of Reproductive Biology. Vol 11. Oxford: Oxford University Press, 1989:61–109.
24. Adams EC, Hertig AT, Rock J. Description of 34 human ova within the first 17 days of development. Am J Anat 1956; 98:435–493.
25. Boué J, Boué A, Lazar P. Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous human abortions. Teratology 1975; 12:11–26.

26. Rogers PAW, Milne BJ, Trounson AO. A model to show human uterine receptivity and embryo viability following ovarian stimulation for in vitro fertilization. J In Vitro Fert Embryo Transf 1986; 3:93–98.
27. Zhou H, Weinberg CR. Evaluating effects of exposures on embryo viability and uterine receptivity in in vitro fertilization. Statist Med 1998; 17:1601–1612.
28. Zhou H, Weinberg CR. Potential for bias in estimating human fecundability parameters: a comparison of statistical models. Statist Med 1999; 18:411–422.
29. Dukic V, Hogan JW. A hierarchical Bayesian approach to modeling embryo implantation following in vitro fertilization. Biostatics 2002; 3:361–377.
30. Speirs AL, Lopata A, Gronow MJ, Kellow GN, Johnston WI. Analysis of the benefits and risks of multiple embryo transfer. Fertil Steril 1983; 39:468–471.
31. Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. NEJM 1999; 340:1796–1799.
32. Rogers P, Leeton J, Cameron IT, Murphy C, Healy DL, Lutjen P. Oocyte donation. In: Wood C, Trounson A, eds. Clinical In Vitro Fertilization and Embryo Transfer. 2nd ed. Berlin: Springer-Verlag, 1988:143–154.
33. Rogers PA, Murphy CR. Uterine receptivity for implantation: human studies. In: Yoshinaga K, ed. Blastocyst Implantation. Serono Symposia. Boston: Adams Publishing Group, 1989:231–238.
34. Cameron IT, Rogers PAW, Caro C, Harman J, Healy DL, Leeton JF. Oocyte donation: a review. BJOG 1989; 96:839–9.
35. Leeton J, Rogers P, King C, Healy D. A comparison of pregnancy rates for 131 donor egg transfers using either a sequential or fixed regime of steroid replacement therapy. Hum Reprod 1991; 6:299–301.
36. Leeton J, Rogers P, Cameron I, Caro C, Healy D. Pregnancy results following embryo transfer in women receiving low-dosage variable-length estrogen replacement therapy for premature ovarian failure. J In Vitro Fert Embryo Transf 1989; 6:232–235.
37. Salat-Baroux J, Cornet D, Alvarez S, et al. Pregnancies after replacement of frozen-thawed embryos in a donation program. Fertil Steril 1988; 49:817–821.
38. Feichtinger W, Kemeter P. Pregnancy after total ovariectomy achieved by ovum donation. Lancet 1985; II:722–723.
39. Asche RH, Balmoceda JP, Ord T, et al. Oocyte donation and gamete intrafallopian transfer in premature ovarian failure. Fertil Steril 1988; 49:263–267.
40. Rosenwaks Z. Donor eggs; their application in modern reproductive technologies. Fertil Steril 1987; 47:895–909.
41. Junca AM, Cohen J, Mandelbaum J, et al. Anonymous and non-anonymous oocyte donation: preliminary results. Hum Reprod 1988; 3:121–123.
42. Devroey P, Wisanto A, Camus M, et al. Oocyte donation in patients without ovarian function. Hum Reprod 1988; 3:699–704.
43. Devroey P, Camus M, van den Abbeel E, van Waesberghe L, Wisanto A, van Steirteghem AC. Establishment of 22 pregnancies after oocyte and embryo donation. BJOG 1989; 96:900–906.
44. Navot D, Laufer N, Kopolovic J, et al. Artificially induced endometrial cycles and establishment of pregnancies in the absence of ovaries. NEJM 1986; 314:806–811.
45. Serhal P, Craft I. Ovum donation—a simplified approach. Fertil Steril 1987; 48:265–269.

46. Serhal P, Craft I. Oocyte donation in 61 patients. Lancet 1989; I:1185–1187.
47. Abdalla HI, Baber RJ, Kirkland A, Leonard T, Studd JW. Pregnancy in women with premature ovarian failure using tubal and intrauterine transfer of cryopreserved zygotes. BJOG 1989; 96:1071–1075.
48. Formigli L, Formigli G, Roccio C. Donation of fertilised uterine ova to infertile women. Fertil Steril 1987; 47:162–165.
49. Navot D, Bergh PA, Williams M, et al. An insight into early reproductive processes through the in vivo model of ovum donation. J Clin Endocrinol Metab 1991; 72:408–414.
50. Tur-Kaspa I, Confino E, Dudkiewicz AB, Myers SA, Friberg J, Gleicher N. Ovarian stimulation protocol for in vitro fertilization with gonadotropin-releasing hormone agonist widens the implantation window. Fertil Steril 1990; 53:859–864.
51. Imbar T, Hurwitz A. Synchronization between endometrial and embryonic age is not absolutely crucial for implantation. Fertil Steril 2004; 82:472–474.
52. Landgren BM, Undén AL, Diczfalusy E. Hormonal profile of the cycle in 68 normally menstruating women. Acta Endocrinol 1980; 94:89–98.
53. Itskovitz-Eldor J, Kol S, Mannaerts B, Coelingh Bennink H. First established pregnancy after controlled ovarian hyperstimulation with recombinant follicle stimulating hormone and the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462). Hum Reprod 1998; 13:294–295.
54. Devroey P, Åbyholm T, Diedrich K, et al. A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist Ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon®). Hum Reprod 1998; 13:3023–3031.
55. Kol S, Lightman A, Hillensjo T, et al. High doses of gonadotrophin-releasing hormone antagonists in in vitro fertilization cycles do not adversely affect the outcome of subsequent freeze-thaw cycles. Hum Reprod 1999; 14: 2242–2244.
56. Albano C, Felberbaum RE, Smitz J, et al. Ovarian stimulation with HMG: results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist cetrorelix and the LHRH-agonist buserelin. Hum Reprod 2000; 15:526–531.
57. Williams SC, Gibbons WE, Muasher SJ, Oehninger S. Minimal ovarian hyperstimulation for in vitro fertilization using sequential clomiphene citrate and gonadotropin with or without the addition of a gonadotrophin-releasing hormone antagonist. Fertil Steril 2002; 78:1068–1072.
58. Barmat LI, Chantilis SJ, Hurst BS, Dickey RP. A randomized prospective trial comparing gonadotrophin-releasing hormone (GnRH) antagonist/recombinant follicle-stimulating hormone (rFSH) versus GnRH-agonist/rFSH in women pretreated with oral contraceptives before in vitro fertilization. Fertil Steril 2005; 83:321–330.
59. Gordon K. Gonadotropin-releasing hormone antagonists implications for oocyte quality and uterine receptivity. Ann NY Acad Sci 2001; 943:49–53.
60. Kol S. Embryo implantation and GnRH antagonists—GnRH antagonists in ART: lower embryo implantation? Hum Reprod 2000; 15:1881–1882.

61. Mannaerts B, Gordon K. GnRH antagonists do not activate the GnRH receptor. Hum Reprod 2000; 15:1882–1883.
62. Diedrich K, Frydman R, Devroey P, et al. Comment on the debate article: Embryo Implantation: the rubicon for GnRH antagonists. Hum Reprod 2001; 16:1305–1306.
63. Kolibianakis E, Albano C, Kahn J, et al. Exposure to high levels of luteinizing hormone and estradiol in the early follicular phase of gonadotropin-releasing hormone antagonist cycles is associated with a reduced chance of pregnancy. Fertil Steril 2003; 79:873–880.
64. Kolibianakis E, Bourgain C, Albano C, et al. Effect of ovarian stimulation with recombinant follicle-stimulating hormone gonadotropin releasing hormone antagonists and human chorionic gonadotropin on endometrial maturation on the day of oocyte pick-up. Fertil Steril 2002; 78:1025–1029.
65. Hernandez ER. Embryo implantation and GnRH antagonists—Embryo implantation: the rubicon for GnRH antagonists. Hum Reprod 2000; 15:1211–1216.
66. Kolibianakis EM, Albano C, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Initiation of gonadotrophin-releasing hormone antagonist on day 1 as compared to day 6 of stimulation: effect on hormonal levels and follicular development in in vitro fertilization cycles. J Clin Endocrinol Metab 2003; 88:5632–5637.
67. Smitz J, Devroey P, Camus M, et al. The luteal phase and early pregnancy after combined GnRH-agonist/HMG treatment for superovulation in IVF or GIFT. Hum Reprod 1988; 3:585–590.
68. Calogero AE, Macchi M, Montanini V, et al. Dynamics of plasma gonadotropin and sex steroid release in polycystic ovarian disease after pituitary–ovarian inhibition with an analog of gonadotropin-releasing hormone. J Clin Endocrinol Metab 1987; 64:980–985.
69. Pritts EA, Atwood AK. Luteal phase support in infertility treatment: a meta-analysis of the randomized trials. Hum Reprod 2002; 17:2287–2299.
70. Daya S, Gunby J. Luteal phase support in assisted reproduction cycles (review). Cochrane Database of Systematic Reviews. Vol. 3. Wiley, 2004.
71. Ragni G, Vegetti W, Baroni E, et al. Comparison of luteal phase profile in gonadotrophin stimulated cycles with or without a gonadotrophin-releasing hormone antagonist. Hum Reprod 2001; 16:2258–2262.
72. Jones GS. Luteal phase defects. In: Behram SJ, Kistner RW, eds. Progress in Infertility. 2nd ed. Boston: Little Brown and Co., 1975:316.
73. Witten BI, Martin SA. The endometrial biopsy as a guide to the management of luteal phase defect. Fertil Steril 1985; 44:460–465.
74. Glazener CM, Kelly NJ, Hull MG. Luteal deficiency not a persistent cause of infertility. Hum Reprod 1988; 3:213–217.
75. Wentz AC, Herbert CM, Maxson WS, Hill GA, Pittaway DE. Cycle of conception endometrial biopsy. Fertil Steril 1986; 46:196–199.
76. Lutjen P, Trounson A, Leeton J, Findlay J, Wood C, Renou P. The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. Nature 1984; 307:174–175.
77. Cameron IT, Rogers PAW, Salamonsen LA, Healy DL. The endocrine requirements for implantation and early embryogenesis. J Reprod Fert Suppl 1988; 36:17–25.

78. Remolí J, Gutierrez A, Cano F, Ruiz A, Simon C, Pellicer A. Long oestradiol replacement in an oocyte donation programme. Hum Reprod 1995; 10: 1387–1391.
79. Ghosh D, De P, Sengupta J. Luteal phase ovarian oestrogen is not essential for implantation and maintenance of pregnancy from surrogate embryo transfer in the rhesus monkey. Hum Reprod 1994; 9:629–637.
80. Li TC, Cooke ID, Warren MA, Goolamallee M, Graham RA, Aplin JD. Endometrial responses in artificial cycles: a prospective study comparing four different oestrogen dosages. Gynaecol 1992; 99:751–756.
81. Zenke U, Chetkowski RJ. Transfer and uterine factors are the major recipient-related determinants of success with donor eggs. Fertil Steril 2004; 82:850–856.
82. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertil Steril 1950; 1:3–25.
83. Lawn AM. The ultrastructure of the endometrium during the sexual cycle. In: Bishop MHW, ed. Advances in Reproductive Biology. Vol. 6. London: Elek, 1973; 61–97.
84. Martel D, Malet C, Gautray JP, Psychoyos A. Surface changes of the luminal uterine epithelium during the human menstrual cycle: a scanning electron microscope study. In: de Brus J, Martel J, Gautray JP, eds. The Endometrium: Hormonal Impacts. New York: Plenum Press, 1981:15–29.
85. Rogers PAW, Murphy CR, Leeton J, Hosie M, Beaton L, Macpherson A. Ultrastructural study of human uterine epithelium from a patient with a confirmed pregnancy. Acta Anat 1989; 135:176–179.
86. Saleh MI, Warren MA, Li TC, Cooke ID. A light microscopical morphometric study of the luminal epithelium of human endometrium during the peri-implantation period. Hum Reprod 1995; 7:1828–1832.
87. Rogers PAW, Hosie MJ, Ortis A, Susil B, Leeton J, Murphy CR. Uterine glandular area during the menstrual cycle and the effects of different in vitro fertilization related hormonal treatments. Hum Reprod 1996; 11:376–379.
88. Creus M, Ordi J, Fábregues, et al. The effect of different hormone therapies on integrin expression and pinopode formation in the human endometrium: a controlled study. Hum Reprod 2003; 18:683–693.
89. Acosta AA, Elberger L, Borghi M, et al. Endometrial dating and determination of the window of implantation in healthy fertile women. Fertil Steril 2000; 73:788–798.
90. Norman RJ, Warnes GM, Wang X, Kirby CA, Matthews CD. Differential effects of gonadotrophin-releasing hormone agonists administered as desensitizing or flare protocols on hormonal function in the luteal phase of hyperstimulated cycles. Hum Reprod 1991; 6:206–213.
91. Murphy CR, Rogers PAW, Leeton J, Hosie M, Beaton L, Macpherson A. Surface ultrastructure of uterine epithelial cells in women with premature ovarian failure following steroid hormone replacement. Acta Anat 1987; 130:348–350.
92. Murphy CR. Understanding the apical surface markers of uterine receptivity—pinopods or uterodomes? Hum Reprod 2000; 15:2451–2454.
93. Nikas G, Drakakis P, Loutradis D, et al. Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. Hum Reprod 1995; 10:1208–1213.

94. Murphy CR, Rogers PAW, Hosie MJ, Leeton J, Beaton L. Tight junctions of human uterine epithelial cells change during the menstrual cycle: a morphometric study. Acta Anat 1992; 144:36–38.
95. Rogers PAW, Murphy CR, Leeton J, Hosie MJ, Beaton L. Turner's syndrome patients lack tight junctions between uterine epithelial cells. Hum Reprod 1992; 7:883–885.
96. Cohen JJ, Debache C, Pigeau F, Mandelbaum J, Plachot M, de Brux J. Sequential use of clomiphene citrate human menopausal gonadotropin and human chorionic gonadotropin in human in vitro fertilization. II. Study of luteal phase adequacy following aspiration of the preovulatory follicles. Fertil Steril 1984; 42:360–365.
97. Abate V, De Corato R, Cali A, Stinchi A. Endometrial biopsy at the time of embryo transfer: correlation of histological diagnosis with therapy and pregnancy rate. J In Vitro Fert Emb Trans 1987; 4:173–176.
98. Birkenfeld A, Navot D, Levij IS, et al. Advanced secretory changes in the proliferative human endometrial epithelium following clomiphene citrate treatment. Fertil Steril 1986; 45:462–468.
99. Sterzik K, Dallenbach C, Schneider V, Sasse V, Dallenbach-Hellweg G. In vitro fertilization: the degree of endometrial insufficiency varies with the type of ovarian stimulation. Fertil Steril 1988; 50:457–462.
100. Garcia JE, Acosta AA, Hsiu JG, Jones HW. Advanced endometrial maturation after ovulation induction with human menopausal gonadotropin/human chorionic gonadotropin for in vitro fertilization. Fertil Steril 1984; 41:31–35.
101. Van Steirteghem AC, Smitz J, Camus M, et al. The luteal phase after in vitro fertilization and related procedures. Hum Reprod 1988; 3:161–164.
102. Macrow PJ, Li TC, Seif MW, Buckley CH, Elstein M. Endometrial structure after superovulation: a prospective controlled study. Fertil Steril 1994; 61: 696–699.
103. Bonhoff A, Naether O, Johannisson E, Bohnet HG. Morphometric characteristics of endometrial biopsies after different types of ovarian stimulation for infertility treatment. Fertil Steril 1993; 59:560–566.
104. Benadiva CA, Metzger DA. Superovulation with human menopausal gonadotropins is associated with endometrial gland-stroma dyssynchrony. Fertil Steril 1994; 61:700–704.
105. Salat-Baroux J, Romain S, Alvarez S, et al. Biochemical and immunohistochemical multiparametric analysis of steroid receptors and growth factor receptors in human normal endometrium in spontaneous cycles and after the induction of ovulation. Hum Reprod 1994; 9:200–208.
106. Rogers PAW, Poison D, Murphy CR, Hosie M, Susil B, Leoni M. Correlation of endometrial histology morphometry and ultrasound appearance after different stimulation protocols for in vitro fertilization. Fertil Steril 1991; 55: 583–587.
107. Fanchin R. Assessing uterine receptivity in 2001. Ultrasonographic glances at the new millenium. Ann NY Acad Sci 2001; 943:185–202.
108. Gonen Y, Casper RF. Prediction of implantation by the sonographic appearance of the endometrium during controlled ovarian stimulation for in vitro fertilization. J In Vitro Fert Emb Trans 1990; 7:146–152.

109. Bustillo M, Krysa LW, Coulam CB. Uterine receptivity in an oocyte donation programme. Hum Reprod 1995; 10:442–445.
110. Fanchin R, Righini C, Ayoubi J-M, et al. New look at endometrial echogenicity: objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization–embryo transfer. Fertil Steril 2000; 74:274–281.
111. Puerto B, Creus M, Carmona F, Civico S, Vanrell JA, Balasch J. Ultrasonography as a predictor of embryo implantation after in vitro fertilization: a controlled study. Fertil Steril 2003; 79:1015–1022.
112. Potlog-Nahari C, Catherino WH, McKeeby JL, Wesley R, Segars JH. A suboptimal endometrial pattern is associated with a reduced likelihood of pregnancy after a days 5 embryo transfer. Fertil Steril 2005; 83:235–237.
113. Coulam CB, Bustillo M, Soenksen DM, Britten S. Ultrasonographic predictors of implantation after assisted reproduction. Fertil Steril 1994; 62:1004–1010.
114. Steer CV, Tan SL, Dillon D, Mason BA, Campbell S. Vaginal color Doppler assessment of uterine artery impedance correlates with immunohistochemical markers of endometrial receptivity required for the implantation of an embryo. Fertil Steril 1995; 63:101–108.
115. Chien L-W, Au H-K, Chen P-L, Xiao J, Tzeng C-R. Assessment of uterine receptivity by the endometrial–subendometrial blood flow distribution pattern in women undergoing in vitro fertilization–embryo transfer. Fertil Steril 2002; 78:245–251.
116. Maugey-Laulom B, Commenges-Ducos M, Jullien V, Papaxanthos-Roche A, Scotet V, Commenges D. Endometrial vascularity and ongoing pregnancy after IVF. Eur J Obstet Gynecol Reprod Biol 2002; 104:137–143.
117. Hung Yu Ng E, Chi Wai Chan C, Shan Tang Oi, Shu Biu Yeung W, Chung Ho P. Comparison of endometrial and subendometrial blood flow measured by three-dimensional power Doppler ultrasound between stimulated and natural cycles in the same patients. Hum Reprod 2004; 19:2385–2390.
118. Schild RF, Knobloch C, Dorn D, Fimmers R, van der Ven H, Hansmann M. Endometrial receptivity in an in vitro fertilization program as assessed by spiral artery blood flow, endometrial thickness, endometrial volume, and uterine artery blood flow. Fertil Steril 2001; 75:361–366.
119. Lesny P, Killick SR. The junctional zone of the uterus and its contractions. BJOG 2004; 111:1182–1189.
120. Liotta L, Petricoin E. Molecular profiling of human cancer. Nat Rev Genet 2000; 1:48–56.
121. Murray MJ, Meyer WR, Zaino RJ. A critical analysis of the accuracy, reproducibility and clinical utility of histologic endometrial dating in fertile women. Fertil Steril 2004; 81:1333–1343.
122. Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. Mol Hum Reprod 2004; 10:879–893.
123. Kao LC, Tulac S, Lobo B, et al. Global gene profiling in human endometrium during the window of implantation. Endocrinology 2002; 143:2119–2138.
124. Borthwick JM, Charnock-Jones DS, Tom BD, et al. Determination of the transcript profile of human endometrium. Mol Hum Reprod 2003; 9:19–33.
125. Carson DD, Lagow E, Thathiah A, et al.Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium

detected by high-density microarray screening. Mol Hum Reprod 2002; 8: 871–879.
126. Riesewijk A, Martin J, van Os R, et al. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Mol Hum Reprod 2003; 9:253–264.
127. Horcajadas JA, Riesewijk A, Polman J, et al. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. Mol Hum Reprod 2005; 11:195–205.
128. Cheng Jr-G, Chen JR, Hernandez L, Alvord WG, Stewart C. Dual control of LIF expression LIF receptor function regulate Stat3 activation at the onset of uterine receptivity embryo implantation. PNAS 2001; 98:8680–8685.
129. Lim H, Ma L, Ma WG, Maas RL, Dey SK. Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidualization in the mouse. Mol Endocrinol 1999:1005–1017.
130. Daftary GS, Taylor HS. Implantation in the human: the role of HOX genes. Semin Reprod Med 2000; 18:311–320.
131. Ye X, Hama K, Contos JJA, et al. LPA_3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. Nature 2005; 435:104–108.
132. Nie G, Li Y, Wang M, Liu YX, Findlay JK, Salamonsen LA. Inhibiting uterine PC6 blocks embryo implantation: an obligatory role for a proprotein convertase in fertility. Biol Reprod 2005; 72:1029–1036.
133. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update 2005; 11:613–630.
134. Tietze C. Reproductive span and rate of reproduction among Hutterite women. Fertil Steril 1957; 8:89–97.
135. Oehninger S, Veeck L, Lanzendorf S, Maloney M, Toner J, Muasher S. Intracytoplasmic sperm injection: achievement of high pregnancy rates in couples with severe male factor infertility is dependent primarily upon female and not male factors. Fertil Steril 1995; 64:977–981.
136. Yaron Y, Botchan A, Amit A, Kogosowski A, Yovel I, Leasing JB. Endometrial receptivity: the age-related decline in pregnancy rates and the effect of ovarian function. Fertil Steril 1993; 60:314–318.
137. Spandorfer SD, Chung PH, Kligman I, Liu H-C, Davis OK, Rosenwaks Z. Clinical assisted reproduction. J Assist Reprod Genet 2000; 17:303–306.
138. Klipstein S, Regan M, Rylcy DA, Goldman MB, Alper MM, Reindollar RH. One last chance for pregnancy: a review of 2705 in vitro fertilization cycles initiated in women age 40 years and above. Fertil Steril 2005; 84:435–445.
139. Navot D, Bergh PA, Williams MA, et al. Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. Lancet 1991; 337:1375–1377.
140. American Society for Reproductive Medicine Guideline for Practice: "Age related infertility" 1995.
141. Abdalla HI, Wren ME, Thomas A, Korea L. Age of the uterus does not affect pregnancy or implantation rates: a study of egg donation of women in different ages sharing oocytes from the same donor. Hum Reprod 1997; 12:827–829.
142. Flamigni C, Borini A, Violini F, Bianchi L, Serrao L. Oocyte donation: comparison between recipients from different age groups. Hum Reprod 1993; 8:2088–2092.

143. Borini A, Bianchi L, Violini F, Maccolini A, Cattoli M, Flamigni C. Oocyte donation program: pregnancy and implantation rates in women of different ages sharing oocytes from single donor. Fertil Steril 1996; 65:94–97.
144. Abdalla HI, Baber R, Kirkland A, Leonard T, Power M, Studd JW. A report on 100 cycles of oocyte donation; factors affecting the outcome. Hum Reprod 1990; 5:1018–1022.
145. Levran D, Ben-Shlomo I, Dor J, Ben-Rafael Z, Nebel L, Mashiach S. Aging of endometrium and oocytes: observations on conception and abortion rates in an egg donation model. Fertil Steril 1991; 56:1091–1094.
146. Cano F, Simon C, Remohi J, Pellicer A. Effect of aging on the female reproductive system: evidence for a role of uterine senescence in the decline in female fecundity. Fertil Steril 1995; 64:584–589.
147. Fitzgerald CT, Seif MW, Killick SR, Elstein M. Age related changes in the female reproductive cycle. BJOG 1994; 101:229–233.
148. Batista MC, Cartledge TP, Zellmer AW, et al. Effects of aging on menstrual cycle hormones and endometrial maturation. Fertil Steril 1995; 64:492–499.
149. Sauer MV, Paulson RJ, Ary BA, Lobo RA. Three hundred cycles of oocyte donation at the University of Southern California: assessing the effect of age and infertility diagnosis on pregnancy and implantation rates. J Assist Reprod Genet 1994; 11:92–96.
150. Check JH, Dietterich C, Lurie M, Nazari A. No evidence of increased uterine vascular impedance with patient ageing following IVF. Hum Reprod 2000; 15:1679–1684.

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Embryo Transfer Technique

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INTRODUCTION

Embryo transfer (ET) is the final, yet crucial step in in vitro fertilization (IVF). Various steps in an IVF cycle can proceed successfully up to the ET stage in about 80% of the cases (1). But, unfortunately, only a small percentage of them achieve pregnancy. The technique of ET has not received enough attention (2,3), as clearly illustrated from the number of scientific publications on ET in the literature relative to the articles published on IVF in general (4). A Medline search revealed that the number of scientific publications on human IVF in the years from 1978–2005 is 16,445. Only 394 articles were directed towards the technique of ET.

Obviously, to most clinicians, the ET technique is a simple procedure. It only means a simple task of inserting the ET catheter in the uterine cavity and ejecting the embryos. Unfortunately, it is not as simple as it looks and it is easier to said than done (5).

The ET technique may directly influence the outcome of assisted reproduction techniques (ART). It has been demonstrated that there is a significant difference in the pregnancy rates associated with different individuals performing the ET within the same IVF program (6,7). In contrast, Visser et al. (8) reported no significant difference among pregnancy rates obtained by three different clinicians. However, when the transfer procedure is standardized, the probability of success in IVF is not dependent on the physician (9). The need to standardize the protocol for ET technique was

regarded as the most important factor influencing the success rate in IVF in a survey of 80 IVF practitioners (10). Moreover, it is estimated that poor ET technique may account for as much as 30% of all failures in assisted reproduction (11). Unfortunately, this failure must have affected thousands of infertile couples seeking pregnancy through assisted reproduction every year.

Therefore, extra attention and time should be given to the procedure of ET. Meldrum et al. (12) recognized that meticulous ET technique is essential for IVF success. This final step in assisted reproduction will determine the fate of a long process and great effort, from ovulation induction and oocytes retrieval, to the tedious high-technology procedures in the laboratory, not to mention the desperate hope of infertile couples.

POTENTIAL NEGATIVE FACTORS ASSOCIATED WITH THE ET TECHNIQUE

ET is routinely performed using the transcervical route, which is basically a blind technique, associated with multiple potential negative factors that may result in total failure of the whole procedure. There is no sure method to confirm that the embryos have been successfully deposited within the uterine cavity and many "lost" embryos may go undetected (3). These potential negative factors include the following.

Initiation of Uterine Contractions

Initiation of uterine contractions that may lead to an immediate or delayed expulsion of the embryos has always been a big concern in assisted reproduction. In an early study on cows, "artificial embryos" in the form of resin spheres impregnated with radioactive gold were traced after ET (13). It was found that after 1.5 hr, a large proportion of the spheres had been expelled from the uterus altogether.

In human IVF, about 15% of the transferred embryos were expelled after the transfer and had been collected from the external cervical os, the tip of the catheter, and the vaginal speculum (14). Similarly, Ménézo et al. (15) were able to demonstrate that only 45% of "experimental embryos" were present within the uterine cavity one hour after the transfer. Experimental studies have been done to demonstrate expulsion of injected material inside the uterine cavity mimicking ET. In a study on humans by Knutzen et al. (16) using radio-opaque dye, mimicking ET, it was found that the dye remained primarily in the uterine cavity in only 58% of cases. It could be concluded from the study that the remainder of the patients would have lost their opportunity for pregnancy as a result of the ET procedure. In a similar study conducted by Mansour et al. (17) using Methylene Blue, it was demonstrated that the dye was visualized at the external cervical os in

42% of the cases, indicating that the uterus extruded the dye at least partially. As a result, it is possible that the embryos may be expelled from the uterus, partially or totally, after the transfer (14,17,18). In a study by Woolcott and Stanger (19), it was observed that the embryos could move as easily toward the cervical canal as toward the fallopian tubes. Fanchin et al. (20) noted that more uterine contractions at the time of transfer were associated with a lower clinical pregnancy rate.

Failure to Pass the Catheter Through the Cervix

It is obviously crucial for the ET catheter to pass the internal os and enter the uterine cavity, otherwise the whole procedure will be a total failure. One cause for the failure of the catheter to pass the internal os is the unnoticed curving of the catheter inside the cervical canal, which can be misleading, especially with soft catheters.

Another important cause for the failure of the catheter to pass the internal cervical os is simply a lack of alignment between the catheter (straight) and the utero-cervical angle (curved or acutely angulated). In extremely rare cases, it is very difficult or even impossible to pass the catheter inside the uterine cavity. This may be due to anatomical distortion of the cervix by previous surgery or fibroid or due to congenital anomaly (4). Scarring of the lower uterine segment or a distorted endometrial cavity create difficulty in catheter introduction (21,22).

Cervical Mucus

Proper embryo replacement can be seriously impaired by cervical mucus. It can plug the tip of the catheter, causing difficulty in delivering the embryos, especially with such a small volume of culture media to inject with the embryos. Plugging the catheter tip can cause embryo retention and damage (especially with assisted hatching) and improper embryo placement (3). Another drawback is the possibility of sticking of the embryos to the mucus around the catheter and dragging them outside during withdrawal of the catheter. Moreover, if the mucus is pushed or injected higher in the uterine cavity, it may interfere with implantation (23).

In a study by Mansour et al. (17), methylene blue was used in a dummy ET model. It was demonstrated that the dye was extruded at the external os in a significantly higher rate when the cervical mucus was not removed. In clinical IVF, in a large study by Nabi et al. (24), it was shown that the embryos were much more likely to be retained when the catheter was contaminated with mucus or blood. Cervical mucus may be a source of bacterial contamination of the embryos and endometrium with subsequent lower pregnancy rates (25,26).

SUGGESTIONS FOR OPTIMIZING THE TECHNIQUE OF ET

Proper Evaluation of the Uterine Cavity

Before starting the IVF cycle, it is important in evaluating the uterine cavity to ensure proper embryo replacement. Proper evaluation can be achieved by the following.

Dummy ET

It has been demonstrated that performing a dummy ET before the IVF cycle significantly improves the pregnancy rate (27). This procedure is important in evaluating the length and the direction of the uterine cavity and cervical canal as well as the cervico-uterine angulations. It also helps in choosing the most suitable kind of catheter to be used. Another advantage of the dummy ET is to discover any unanticipated difficulty in introducing the catheter such as pinpoint external os, the presence of cervical polypi or fibroids, or anatomical distortion of the cervix from previous surgery or congenital anomaly. If cervical stenosis is diagnosed, it is advisable to perform cervical dilatation before starting the IVF cycle (28,29). The use of cervical laminaria one month before the IVF cycle has been demonstrated to be beneficial as a means of cervical dilatation (30). The procedure of dummy ET is recommended to be done one to two months before the start of the IVF cycle (3,27) or immediately before the actual ET. It is recommended to perform both.

Ultrasonographic Evaluation

Another important method for evaluating the uterine cavity is using ultrasound (US). The length of the uterine cavity and the cervical canal can be precisely measured. The use of US to determine the accuracy of trial ET was studied by Shamonki et al. (31). It was demonstrated that approximately 19% of patients had a discrepancy of $\geq$1.5 cm and approximately 30% had a difference of $\geq$1 cm from trial ET compared of US-guided ET, suggesting a benefit of US-guided ET. Ultrasonography is very important in measuring and evaluating the cervico-uterine angle (4,32). Revising the US picture of the uterine cavity, length, and direction before the ET functions as a map or a guide before performing the transfer, which is essentially a blind technique. Ultrasonography is also very important in diagnosing fibroids that may be encroaching on the uterine cavity or distorting the cervical canal, as well as diagnosing any uterine anomalies.

Avoiding the Initiation of Uterine Contractions

The demonstration of endometrial movements (33) has opened a research field that may influence the outcome of assisted reproduction.

Every precaution has to be taken to avoid the initiation of uterine contractions.

Avoid Touching the Uterine Fundus

It is observed by most gynecologists that if the tip of the catheter touches the uterine fundus, the patients experience immediate discomfort followed by suprapubic pain or heaviness. This is probably associated with the initiation of uterine contractions. It was demonstrated that touching the fundus with the catheter stimulated uterine contractions (20,34). Using US visible material in a mock transfer by Lesny et al. (34) demonstrated that touching the fundus with the catheter initiated strong random uterine contractions and the contrast material was relocated from the fundus in six of seven patients. Early sources in IVF described the optimal location for embryo placement as between 0.5 and 1.0 cm from the fundus (35,36). Not touching the fundus was ranked high as a prognostic factor for IVF success in a survey done by Kovacs (2).

To avoid touching the fundus, some IVF specialists routinely place the catheter approximately 0.5 cm below the fundus (37,38) or 1–1.5 cm from the fundus (39). Depositing the embryos in the mid-fundal area of the uterus was found to be important in improving the pregnancy rate (40–44). Waterstone et al. (40) reported a significant increase in pregnancy rate by changing the position of the catheter so as to avoid touching the uterine fundus. Coroleu et al. (45) demonstrated that the position of the ET catheter 2 cm from the fundus was superior to 1 cm from the fundus. In a study by Frankfurter et al. (43), the authors demonstrated that the implantation rate as well as the pregnancy and birth rates were significantly higher after middle to lower uterine segment ET compared with fundal ET.

Therefore, individual measurement of the cervical canal and the uterine cavity length are extremely important. It could be done previously during the dummy ET or by US evaluation of the uterine dimensions. However, it has been demonstrated that the use of a fixed-distance technique greatly reduced the variation in pregnancy rates among physicians (46), probably due to reduction in the rate of touching the fundus as when the clinical touch method was used.

Catheter Type

Since the beginning of IVF, the value of soft ET catheters has been recognized. The ideal ET catheter should be soft enough to avoid any trauma to the endocervix or endometrium and malleable enough to find its way through the cervical canal into the uterine cavity (4). The word "soft" means a combination of physical flexibility, malleability, and smoothness of the tip (11). It is worth mentioning that in order to benefit from the advantages of the softness of the catheter, the outer rigid sheath should be minimally used to stop short of the internal cervical os. If the outer sheath is introduced

first, it will convert a "soft" catheter into a "stiff" catheter (3). The stimulus of the ET catheter passing through the internal cervical os can also initiate contractions, which are probably mediated by the release of prostaglandins (47). The mere presence of the ET catheter inside the cervical canal and uterine cavity might be one of the factors that can trigger uterine contractions (48).

Several studies have compared different kinds of catheters and found improved pregnancy rates with soft catheters (11,27,49,50). Other groups found no difference (37,39,51–55). In a large prospective randomized study (56), ET using a soft catheter was compared to a rigid catheter. The results demonstrated that the ongoing pregnancy rate was significantly higher in the soft catheter group. Similarly in another randomized trial comparing soft ET catheter versus firm ones, the pregnancy rate was increased by 50% when soft catheters were used (57). Changing from rigid to soft catheters has been associated with an improvement in pregnancy rates (41,58). The soft catheter was found to be superior to the stiffer catheter when transferring embryos subjected to assisted hatching (49). A recent meta-analysis of 10 studies comparing soft ET catheters with more rigid ones revealed that the clinical pregnancy rate was significantly better using the soft catheters (Fig. 1) (59).

Gentle Manipulation

As a general rule, the ET procedure should be a simple and painless procedure. Atraumatic delivery of embryos into the endometrial cavity is the prime goal of ET (3). Gentle manipulation throughout the total procedure

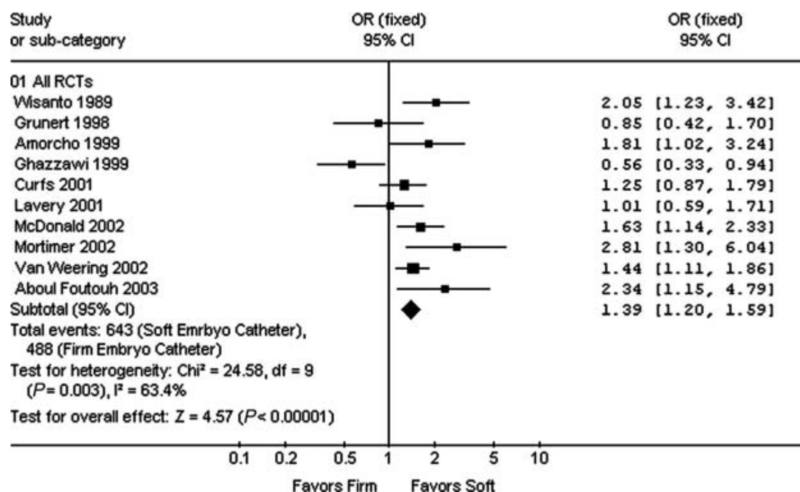

Figure 1 Meta-analysis comparing in vitro fertilization outcome using soft versus firm embryo transfer catheters.

of the ET should be the rule, even in introducing the vaginal speculum to avoid unnecessary pushing of the cervix. It was observed that just simple insertion of the catheter results in uterine movement (16). Obviously, using a volsellum to hold the cervix should be avoided except in rare cases (2). Stimulation of the cervix results in the release of oxytocin, thus increasing uterine contractility. In a prospective clinical study on humans, serial blood samples were collected in time intervals of 20 sec during the ET procedure in order to measure serum oxytocin concentration (60). It was demonstrated that in the absence of a tenaculum placement, no increase in oxytocin concentration was observed. When a tenaculum was used, it was temporarily associated with an elevation in oxytocin levels, which remained elevated until the end of the ET procedure. The use of tissue forceps to hold the cervix has been observed to trigger uterine contractions (61). In some cases of difficult ET, applying 1–2 mL of local anesthetic (1% procaine) to the anterior lip of the cervix through a very fine needle and then applying a tenaculum was found to be very acceptable to the patients, caused no discomfort, and did not affect the outcome (39).

A number of studies have shown that technically difficult ETs are associated with reduced pregnancy rates (8,27,48,62–64). It probably could be due to the initiation of uterine contractions that expel the embryos (20). It has been shown that in 87% of the achieved pregnancies, the ET was performed atraumatically and without bleeding (37). The presence of blood in the catheter was found to be associated with decreased implantation and pregnancy rates (65). A recent report of 30 case series using direct hysteroscopic visualization to assess the effects of ETs on endometrial integrity showed that clinical perception of the ease of transfer does not correlate well with the degree of endometrial disruption (66).

Uterine Relaxing Substances

Many researchers have given different drugs in an attempt to decrease uterine contractions during ET.

Serum progesterone levels on the day of ET correlate with the frequency of uterine contractions which decreased as the level of progesterone increased (20). Progesterone was administered starting on the day of oocytes pickup to relax uterine contractility at the time of ET (67,68). Starting progesterone administration on the day of pickup did not have additional improved PR as compared to starting it on the day of ET (69).

Nonsteroidal anti-inflammatory drugs (NSAIDs) block the action of cyclooxygenase (COX) and inhibit the production of prostaglandin (70). Accordingly, 10 mg of the NSAID piroxicam was given in a prospective randomized study one to two hours before ET. The results demonstrated significant improvement of implantation and pregnancy rates with the use of piroxicam (71).

Sedation with 10 mg valium, 30 minutes to 1 hour before ET, was a common practice (36); however, it did not make any difference (37).

Tacolytic agents or prostaglandin synthetase inhibitor did not have a significant effect (37).

The use of Propofol general anesthesia for ET did not have a significant effect (72). However, it could be used in some patients who experience severe stress and anxiety during ET.

Ensuring the Passage of the ET Catheter into the Uterine Cavity

The ultimate goal at the end of an IVF cycle is to safely deposit the embryos inside the uterine cavity. One has to be absolutely sure that the catheter has passed the internal os and has not kinked or curved inside the cervical canal. Soft catheters can sometimes be misleading. This can be discovered by experienced practitioners and by doing the simple test of rotating the catheter by 360°. If it recoils, it means it is curved inside the cervical canal. Choosing the most suitable catheter for each patient should be done before the actual ET by performing a dummy ET, to avoid harshly navigating the cervical canal with the ET catheter loaded with embryos. Neithardt et al. (73) placed the ET catheter first and then the embryo after "loading" was done.

One of the most important causes of failure to pass the catheter into the uterine cavity is simply the pronounced curvature or angulations of the cervico-uterine angle (Fig. 2). Proper evaluation of the cervico-uterine angle and determining how much curvature is needed for the catheter should be done before loading the embryos. A situation in which you need to curve the catheter while you have the embryos loaded should be completely avoided. That is why it is important to perform a dummy ET right before the actual one and revise the previously performed US picture of the uterus. It has been demonstrated that molding the ET catheter according to the cervico-uterine angle by US improved the clinical pregnancy and implantation

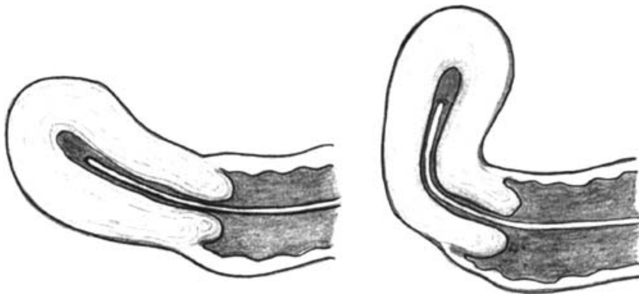

Figure 2 Curving a rigid but malleable embryo transfer catheter according to the cervico-uterine angle overcomes almost all difficult cases.

rates and diminished the incidence of difficult transfers (32). Straightening the utero-cervical angle can be achieved with a full bladder before ET (63,74). This effect is being achieved indirectly by performing ET under US guidance (74,75).

Another method to simply facilitate entering the catheter is to gently maneuver the vaginal speculum (the degree of opening and how far it is pushed inside).

The use of a more rigid catheter is sometimes needed in order to pass the internal os. It is advantageous for these rigid catheters to be malleable. Malleability is essential to allow making the required curve, which will overcome the acute cervico-uterine angulations. Using a malleable stylette to place the outer sheath correctly and negotiate the cervical canal before introducing the soft catheter was found to have no negative impact on implantation and delivery rates (76).

The use of special introducers designed to overcome difficulties in selected patients in passing the ET catheter was described (77).

Holding the cervix with a volsellum in order to stabilize the uterus while introducing the catheter should be rarely resorted to. The effect of cervical traction with a tenaculum on the utero-cervical angle was studied using radio-opaque guide-wire (78). The authors found that moderate cervical traction straightens the uterus. It was concluded that routine use of the tenaculum theoretically makes the passage of an ET catheter easier and less traumatic. On the other hand, it should be remembered that holding the cervix with a volsellum results in the release of oxytocin (60) and initiates uterine contractions (61). Moreover, holding the cervix with a volsellum is painful and should be done under general anesthesia (72) or local anesthetic (39).

In some rare cases, it is difficult or even impossible to pass the catheter inside the uterine cavity. For these cases, stiffer and more rigid catheters may be used (27,63). Another system that has been used in these difficult cases is the coaxial catheter (21). Canulation of a resistant internal os with the malleable outer sheath of a coaxial soft ET catheter did not affect IVF outcome (79). A malleable stylette can be used to place the outer sheath of a soft catheter past the internal os. The stylette is then removed and the inner clear catheter loaded with the embryos is inserted. Using this technique, pregnancy rates equivalent to easy transfer were obtained (80).

Hysteroscopic evaluation and/or correction of the endocervix, followed by transcervical placements of a Malecot catheter for an average of 10 days, is a technique that allowed easier entry through the cervical canal in patients for whom previous ET has been difficult (81). Cervical dilatation may be resorted to in cases of cervical stenosis. A short interval between dilatation and ET is not recommended (3). Very low pregnancy rates were reported when cervical dilatation was done during oocyte pickup or two days before ET (8,82). Performing cervical dilation before the start of IVF cycle resulted in easier transfer and improved pregnancy rates (28,29). It is

also helpful to place a laminaria approximately one month before starting the IVF cycle (30) or to place hygroscopic rods in the cervix prior to ovarian stimulation (83). The standard method of dilatation with successively larger dilators may be difficult and traumatic in some tortuous or stenotic cervical canals. Canulation of the cervix under fluoroscopic guidance and dilating the endocervical canal was successfully tried (22).

Sometimes it is even impossible to perform transcervical ET. In an early report in 1985, in 867 ET procedures, 1.3% were impossible, 3.2% were very difficult (manipulation for > 5 min or cervical dilatation), and 5.6% were difficult (84). Twenty years after this report, the current experience of most IVF centers makes the rate of impossible and difficult ET procedures significantly less. Very rarely, when other maneuvers fail, trans-myometrial surgical ET can be used (82,85,86). Surgical transfer of the embryos through the fundus has been tried, originally to avoid the initiation of uterine contractions induced by passing the catheter through the cervix (87). In 1987, Lenz and Leeton (88) suggested US-guided transabdominal transvesical and transfundal ET; however, none of the 10 patients conceived. Ultrasonic transvesical transmyometrial ET technique has been proposed by Wikland et al. (89); however, no improved pregnancy rate was achieved over the transcervical route. Surgical ET has been used successfully, achieving results comparable to the transcervical route (90). This technique is straightforward and requires no greater expertise than that necessary for US-guided oocyte collection and requires no sedation nor anesthetics (90). The surgical ET set is composed of a metal needle (like an oocyte pickup needle) with a stellet and an ET catheter that fits in the needle after withdrawal of the stellet. Under US guidance, the needle is introduced transmyometrially and the tip is stopped in the endometrium just beneath the cavity. At this time, the stellet is replaced with the ET catheter and the embryos are ejected, then the whole set is withdrawn. The results of a small prospective study showed no benefit to electing transmyometrial ET in preference to transcervical ET in patients who had previously failed IVF (82).

Getting Rid of Cervical Mucus

Cleaning the cervix of the cervical mucus before ET is advisable to avoid the possible drawbacks mentioned previously. Removing the cervical mucus before ET can be done by repeated gentle aspiration using a 1 cm^3 syringe with its tip placed at the external os or using a soft catheter attached to a syringe. The endo-cervix can be cleaned of mucus using a cotton swab and sterile saline initially, then a small amount of culture media (8,39). Repeated irrigation of the cervical canal with 3 mL culture medium and aspiration of the mucus has been described by Silberstein et al. (79). Vigorous cervical lavage before ET was evaluated by McNamee et al. (91) in a retrospective study and was found to improve the pregnancy rate. In a

randomized controlled study by Sallam et al. (92), no significant difference was found in pregnancy rates with or without flushing. Also, a large multicenter study did not show a significant difference (93).

Prevention of Embryo Expulsion After ET

A technique using the vaginal speculum to prevent embryo expulsion after ET was recently described (94). The idea is a simple one depending on applying gentle pressure on the cervix using the vaginal speculum (Fig. 3). In summary, after introducing the ET catheter and stopping short of the fundus, the screw of the vaginal speculum is loosened so that its two blades collapse on the cervix applying gentle pressure and occluding the cervix. After waiting for one minute, the embryos are ejected and the catheter is withdrawn slowly. The speculum is kept in place for an average of seven minutes and then removed. Using this technique, the implantation and clinical pregnancy rates were significantly improved.

Other Factors in ET Technique That May Affect the IVF Outcome

ET Under Ultrasonographic Guidance

Performing the ET procedure under US guidance is another way to ensure that the ET catheter has passed internal os and entered the uterine cavity. Various groups described the use of US to facilitate ET (95–97) and it has proven useful in women with previously difficult ET (98). Using US guidance for ET was demonstrated by a number of studies to be simple and reassuring, and it significantly improved pregnancy rates by optimizing placement of the embryos (11,50,99–105). However, other groups found no significant difference in the pregnancy rates when ET was performed under US-guidance and clinical touch ET (39,98,106). A major factor in

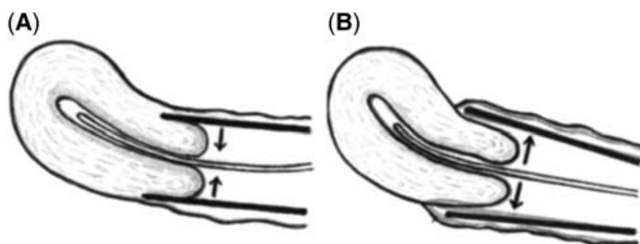

Figure 3 (**A**) In the study group, the two valves of the vaginal speculum are closed on the cervix. (**B**) In the control group, the two valves of the vaginal speculum are open.

making the difference between US-guided ET and clinical touch ET is the experience of the practitioner. For easier identification of the ET catheter, some kinds have an ultrasonically visible echo-tip (54) or the presence of air bubbles (39). A meta-analysis was done for eight prospective controlled studies comparing US-guided and clinical touch ET (107). The results showed that US-guided ET significantly increased the implantation and clinical pregnancy rates. It was reported recently that transrectal US was used successfully during ET in obese women (108).

Loading of the ET Catheter

Loading of the ET catheter should start after cleaning the cervix of cervical mucus and performing the dummy or trial ET. According to the dummy ET, the suitable ET catheter is selected and flushed with tissue culture medium. After flushing, the ET catheter is filled with culture medium with an extra 10–15 μL in the attached syringe. The required embryos for ET are aspirated in another 10 μL medium and a final 10 μL medium is aspirated to move the embryos away from the catheter tip (Fig. 4). Using a continuous fluid column containing embryos near the tip is recommended (3,100). The quantity of fluid used for ET should be as small as possible to prevent the embryos from flowing out of the cervical canal or into the tubes (37). A large volume (60 μL) of transfer media and a large air bubble in the catheter may result in the expulsion of the embryos (14). Meldrum et al. (12) demonstrated an improvement in the pregnancy rates after reducing the total transfer volume. A continuous fluid column of 30 μL is recommended (3).

Air loaded into the transfer catheter to bracket the embryo-containing medium was a standard practice (8,39,43,79,87,109–111). Two prospective randomized studies were done to investigate the effect of the presence of air bubbles in the ET catheter on the outcome of IVF (112,113). The authors concluded that the presence of air bubbles has no negative effect on ET success. In an experimental study using Methylene Blue dye (17), the presence of air bubbles did not significantly affect the rate of extrusion of the dye at the external cervical os. However, there is no definitive reason to support the

(A)

(B)

Figure 4 Loading of the embryo transfer catheter: (**A**) continuous fluid column of tissue culture media containing the embryos and (**B**) the media containing the embryos between two air brackets.

enclosure of air spaces before and after the medium containing the embryos in the ET catheter. It was suggested that the air bubbles mark the position of the embryos inside the catheter (112) and protect against loss (114) or entangling with mucus (112) and cling in the catheter during withdrawal (109).

The concentration of protein in the transfer media does not affect the results (115), nor does increasing the viscosity (116).

Retained Embryos

One of the problems of ET is finding retained embryos in the catheter after the procedure, which decreases the implantation rate (8,65). This problem occurs much more frequently after a difficult ET (24). However, the pregnancy rate was not compromised when the retained embryos were returned immediately (24,117,118). Embryos were significantly more likely to be retained when the transfer catheter was contaminated with mucus (17.8 vs. 3.3%) or blood (12 vs. 3.3%) (119).

In a retrospective analysis of 1363 ET procedures, it was found that 3.9% of all transfers were complicated by finding retained embryos (120). The authors concluded that immediate transfer of embryos retained in the catheter following the initial transfer attempt did not have an adverse effect on pregnancy outcome. However, it has been shown previously that the approach of immediately retransferring retained embryos does not solve the problem (8), and they suggested that ET should be repeated one day later.

Another possible cause for retained embryos is the position of the embryos in the catheter. Small volumes of less than 40 μL are preferable, but it is advisable to aspirate approximately 15–20 μL of culture medium first, and then the embryos are aspirated second. This is to ensure enough media to push out the embryos (4). It is also important, once the injection is done, to keep the pressure on the plunger of the syringe until complete withdrawal of the catheter (7) to avoid re-aspirating the embryos. Some syringes have the property to "recoil" that causes respiration of the embryos back in the catheter when the plunger is released (3).

An important precaution to minimize retained embryos is a slow withdrawal of the catheter after ejecting the embryos. Rapid withdrawal may create negative pressure and result in the withdrawal of the embryos following the catheter. It was found by Leong et al. (96) that withdrawal of the catheter about 1 cm and brisk injection avoided retrograde flow of the transfer media along the catheter by "capillary action."

Position of the Patient and Bed Rest After ET

The patients usually mistakenly believe that they can contribute to the success of their IVF cycle through their bed position and the amount of bed rest.

Theoretically, if the uterus is anteverted it seems preferable to perform ET in the knee chest position. However, in a randomized study there was no

difference in the pregnancy rate according to the position of the patients during ET (37). Most of IVF centers use the lithotomy position or lithotomy in a slightly trendelenburg position (39).

Bed rest after ET was originally practiced in most IVF centers for several hours possibly because of fear of mechanical expulsion of the embryo (23,121–123). In 1993, Al-Shawaf et al. (39) and in 1995 Sharif et al. (63) have suggested that there is no justification for bed rest after ET. Then, a randomized prospective controlled study by Botta and Graudzinskas (124) demonstrated that there was no benefit of bed rest after ET. In a large study with a historical cohort–control (125), there was no benefit of bed rest following ET. The position of the embryos after ET immediately in a standing position was ultrasonically tracked by Woolcott and Stanger (126). It was demonstrated that standing shortly after ET does not play a significant role in the final position of the embryos. It has also been shown recently in a prospective study of 406 patients that immediate ambulation following the ET has no adverse effect on the pregnancy rate as compared to bed rest for one to two hours (127).

It is worth mentioning that the so-called endometrial cavity is a potential space and not a real one. The ET catheter only separates the opposed endometrial surfaces and, once the catheter is removed, the endometrial surfaces re-oppose. The embryos and fluid injected into this potential space are then relocated by the endometrial and myometrial peristalsis as well as the surface tension between the fluid–solid interface (33,126,128). It is believed that the embryos generally implant where they are deposited and it confirms the importance of careful embryo placement (3). Mock ET was performed using small microspheres immediately before hysterectomy. The uterine cavity was then inspected and the microspheres were found within 1 cm of the site of deposition (129). In a study by Baba et al. (130), it was found that 26 of 32 gestational sacs, seen by three-dimensional US, were in the area where the air bubble was seen immediately after transfer.

Withdrawal of the Catheter After ET

The time interval between deposition of the embryos and withdrawal of the catheter has been investigated by many researchers. Waiting before withdrawal of the catheter was suggested so that the uterus can stabilize (51). The catheter was kept in place for one minute before the embryos were gently injected, then a further one minute waiting period followed before the catheter was slowly withdrawn. Based on a mock ET and visualization of radio-opaque dye, it was recommended to routinely wait 15 seconds after introducing the ET catheter into the uterus before ejecting the embryos and another 60 seconds before catheter removal (16). Zeck et al. (131) recommended immediate withdrawal of the catheter after ET. However, Martinez et al. (132) demonstrated an improved PR with a delay of 30 seconds before

withdrawing the catheter. Negative pressure of capillary action created by withdrawing the catheter could draw embryos into the cervical canal (3).

Intrafallopian ET

Hypothetically, the intrafallopian environment is more physiological to the embryos after IVF. Based on this assumption, tubal transfer was proposed by some investigators (133,134). Naturally, the fallopian tubes have to be healthy. It was found to be promising in cases of male factor infertility (37).

A limited number of investigators have proposed intrafallopian ET as an alternative to the transcervical route. Using this technique through laparoscopy in 10 patients, six patients achieved pregnancies (135) and five patients out of 10 (136). Retrograde tubal transfer was also described in 28 patients and five pregnancies resulted (137). However, for practical reasons and based on the success rates, the transcervical ET is the routine procedure used by all IVF centers.

ET Technique as a Cause of Ectopic Pregnancy

The risk of ectopic pregnancy following IVF was found to be 5% in a multicenter study undertaken on 1163 pregnancies (138). This figure is certainly higher than that in natural conception.

The influence of transfer distance from the fundus on the clinical pregnancy rate and ectopic pregnancy rate was investigated by Pope et al. (139). The authors demonstrated that increasing the transfer distance from the fundus significantly increases the PR and lower the ectopic rate. The results suggested that for every additional millimeter embryos are deposited away from the fundus, the odds of clinical pregnancy are increased by 11%. ET technique as a cause of ectopic pregnancy was recognized early by Yovich et al. in 1985 (110). The authors reported an incidence more than three times greater in ectopic pregnancy when the embryos were transferred near the fundus as compared to the mid-cavity position. They concluded that the catheter need to be inserted only 55 mm as a routine and less in patients with a shortened cervix or with hypoplastic uterus (110). The midfundal technique resulted in a lower percentage of ectopic pregnancies and did not negatively affect the pregnancy rate (140). Transferring the embryos by replacement at 6 cm without tracing the position of the fundus was also demonstrated to improve the pregnancy rates (5). It was reported that two cases of cervical pregnancy resulted from ET to the lower uterine cavity (141).

Ectopic pregnancy was found to be 3.9 times more frequently associated with difficult ET than with an easy procedure (142). Moreover, intramural pregnancy was reported following difficult ET (143).

The size of the uterus was shown to be a critical factor in the etiology of ectopic pregnancy in IVF (38). The study demonstrated that the ectopic pregnancy rate was significantly higher in women with uterine cavity length

less than 7 cm. Uterine contractions in the early luteal phase are generally cervico-fundal in origin (142) and it may be the cause for some ectopic pregnancy in IVF (3).

Bacterial Contamination

Cervical infection was found to cause diminished pregnancy and implantation rates. It was found that cervical mucus tested positive in culture in 71% of patients and 49% of patients had positive culture of the catheter tip (25). The clinical pregnancy rate was significantly reduced in catheter tip-positive patients. Similarly, Fanchin et al. (26) demonstrated a significant reduction in the pregnancy rates in positive culture patients. A meta-analysis of controlled studies found that the clinical pregnancy rates and implantation rates were significantly diminished in the presence of cervical infection (144).

Pelvic infection is likely to complicate ET. A pelvic abscess was reported after transcervical ET in an oocytes donation recipient (145). However, the value of routine administration of antibiotics before the IVF cycle or following oocyte retrieval or ET has not been evaluated by randomized controlled studies.

DESCRIPTION OF AN ET PROCEDURE

The patient is instructed to come fasting as a precaution in case the need for general anesthesia arises.

The patient is informed of the fertilization rate, the number of available embryos, and the number of embryos selected for the transfer.

The patient is assured that the ET is a simple procedure. If she is very stressed, it is better to perform ET under general anesthesia.

The previously taken US picture of the uterus and the dummy ET is revised to get an idea about the length and direction of the uterus and the degree of cervico-uterine angulation.

The patient is put in the lithotomy position and the cervix is visualized using Cusco's speculum.

The cervix and the vaginal vaults are cleaned of cervical mucus and vaginal secretions using tissue culture media and sterile gauze.

The cervical mucus at the external os is aspirated gently and repeatedly using a 1 cm^3 syringe.

A dummy ET is done using a sterilized soft ET catheter. The soft inner catheter is advanced from the outer rigid sheath and introduced through the cervical canal to pass the internal cervical os and enter the uterine cavity. The outer rigid sheath is stopped short of the internal os. The catheter is rotated by 360° and leave it, if it recoils, it means it is kinked inside the cervical canal and did not pass the internal os. The catheter is withdrawn and tried again after changing the position of the speculum (degree of opening

and how far it is introduced). If the soft catheter failed to be introduced, a more rigid but malleable catheter is tried. The catheter is curved according to the curvature of the cervico-uterine angle seen in the US picture. The curved catheter is introduced gently to follow the curvature of the cervix and it is moved in different directions until it passes the internal os. Sometimes you need to increase the curvature of the catheter in order to overcome the acute angulations of the cervico-uterine angle. In almost all cases, it is possible to introduce the rightly curved rigid catheter.

In the case of a failed dummy ET, the procedure is stopped and the patient is transferred for general anesthesia.

If the dummy ET catheter was successfully introduced, the actual ET can be started. The catheter type is chosen according to the suitable one used for the dummy trial. The ET catheter is flushed with tissue culture medium, and then filled with the transfer medium. About 15 μL of transfer medium is aspirated first and then the embryos are aspirated next in another 10 μL medium. Finally, 10 μL medium is aspirated to withdraw the embryos away from the catheter tip.

The loaded ET catheter is introduced through the cervix to pass the internal os and then gently advanced in the mid-uterine cavity and stopped from 1–2 cm short of the fundus.

The screw of the vaginal speculum is loosened so that the two valves of the vaginal speculum apply a gentle pressure on the portio-vaginalis.

At this moment, some patients experience suprapubic heaviness and discomfort. After one to two minutes, when this complaint disappears, the embryos are ejected and pressure is kept on the plunger of the syringe while slowly withdrawing the catheter out. The speculum is kept in place for an average of seven minutes and then removed.

The catheter is checked for any retained embryos. If found, retransfer is done immediately.

For difficult cases, general anesthesia is given in the form of propofol 2 mg/kg as an induction dose and anesthesia is maintained by inhalation of isoflurosane 1.5% and oxygen 100% through a facemask. The dummy ET is repeated and if not successful a tenaculum is used to stabilize the cervix. As a last resort, a special rigid but malleable introducer may be used. In extremely rare cases, transmyometrial surgical ET may be resorted to.

LUTEAL PHASE SUPPORT

When Edwards and Steptoe started clinical IVF, human oocytes were successfully fertilized and grown in vitro; however, no pregnancy resulted for the first seven years. It was then realized that the failure of embryos to implant was due to luteal phase disruption (146).

Normal luteal function is important for supporting pregnancy. The aspiration of granulosa cells during oocyte pickup can interfere with

the production of progesterone (147), although not consistently (148). It has been demonstrated that removal of the corpus luteum during early pregnancy resulted in complete abortion (149,150).

The use of GnRH agonist (GnRHa) in stimulation protocols for IVF has dominated all other protocols and is being used by almost all IVF centers (151,152). The agonist may create luteal phase defect (153) due to impairment of the ability of corpus luteum to produce progesterone (154), thus luteal phase support was considered essential (155). Recently, GnRH antagonists are used with increasing frequency in ART cycles. It has been demonstrated that luteal function is less impaired in GnRH antagonist than in GnRHa treatment (156). The question of the need to supplement the luteal phase after the use of GnRH antagonist needs further investigation.

All systematic reviews and meta-analysis have confirmed the importance of luteal phase support in ART (157–160). Progesterone administration was found to significantly improve the fertility outcomes in ART compared to no treatment (157). Progesterone was found to be equivalent to the use of human chorionic gonadotrophin (hCG) for luteal phase support but had a decreased incidence of OHSS (159,161).

The most commonly used method of administration of progesterone is IM injection. It was found to be associated with normal endometrial response (162). Intramuscular progesterone luteal supplementation versus no treatment or placebo resulted in significantly better clinical pregnancy rate and delivery rate (163,164). Compared to vaginal gel or vaginal cream preparations of progesterone, IM injection resulted in a significantly improved clinical pregnancy and delivery rates (163–166).

The dose of IM progesterone injection is 50–100 mg/day. In one study (167), daily injection of 50 mg progesterone was compared with injection of 341 mg of 17α-hydroxyprogesterone caproate every three days and there was no significant difference in the clinical pregnancy rate or abortion rate. Daily IM injection with 25 and 100 mg progesterone was compared, and no significant difference in the clinical pregnancy rate or delivery rate was found (168). Oral or vaginal progesterone is given in the form of a micronized preparation of 400–600 mg/day (158).

The duration of luteal phase supplementation is variable in different studies, from supplementation for two to three weeks only and through 10 to 12 weeks of gestation (158).

Local and systemic allergic reactions to the oil in IM injections of progesterone may result (161). Two cases of acute easinophilic pneumonia following IM administration of progesterone were reported (169).

Oral progesterone administration was found to be associated with significantly lower implantation and pregnancy rates, high miscarriage rates, or both compared with IM or vaginal route (170,171).

The addition of estrogen to the standard progesterone in the luteal phase resulted in significantly higher implantation and pregnancy rates

compared with the use of progesterone alone (172). Estrogen doses varied from 2 to 6 mg oral per day (158). Phytoestrogens were also found to significantly improve the implantation rates, clinical pregnancy rates, and delivery rates when added to progesterone as compared with progesterone alone (173).

In conclusion, the routine use of GnRH agonist in IVF cycles creates persistent LH suppression and luteal phase defect. Luteal supplementation with IM progesterone or IM hCG significantly improved the clinical pregnancy rates and delivery rates compared with no treatment (157). There is no difference between IM hCG and IM progesterone, however, hCG is associated with a significantly higher OHSS (160). The most benefit is obtained when progesterone is given IM as compared to oral or vaginal use. Addition of estrogen to progesterone improved the implantation rate (158).

REFERENCES

1. Human Fertilisation and Embryology Authority, Fifth Annual Report. London: Human Fertilisation and Embryology Authority, 1996.
2. Kovacs GT. What factors are important for successful embryo transfer after in vitro fertilization? Hum Reprod 1999; 14:590.
3. Schoolcraft WB, Surrey ES, Gardner DK, Phil D. Embryo transfer: techniques and variables affecting success. Fertil Steril 2001; 5:863.
4. Mansour RT, Aboulghar MA. Optimizing the embryo transfer technique. Hum Reprod 2002; 17:1149.
5. Naaktgeboren N, Dieben S, Heijnsbroek I. Embryo transfer easier said than done. Fertil Steril 1998; 70:S352.
6. Karande VC, Morris R, Chapman C, Rinhart J, Gleicher N. Impact of the 'Physician factor' on pregnancy rates in a large assisted reproductive technology program: do too many cooks spoil the broth? Fertil Steril 1999; 71:1001.
7. Hearns-Stokes RM, Miller BT, Scott L, Creuss D, Chakraborty PK, Segars JH. Pregnancy rates after embryo transfer depend on the provide at embryo transfer. Fertil Steril 2000; 74:806.
8. Visser DS, Fourie F, Kruger HF. Multiple attempts at embryo transfer: effect on pregnancy outcome in an in vitro fertilization and embryo transfer program. J Ass Reprod Genet 1993; 10:37.
9. van Weering HG, Schats R, McDonnell J, Hompes PG. Ongoing pregnancy rates in in vitro fertilization are not dependent on the physician performing the embryo transfer. Fertil Steril 2005; 83:316.
10. Salha OH, Lamb VK, Balen AH. A postal survey of embryo transfer practice in the UK. Hum Reprod 2001; 4:686.
11. Cohen J. Embryo replacement technology. San Francisco 31 Annual Post Graduate Course, ASRM, 1998.
12. Meldrum DR, Chetkowski R, Steingol KA, de Ziegler D, Cedars MI, Hamilton M. Evolution of a highly successful in vitro fertilization–embryo transfer program. Fertil Steril 1987; 48:8.
13. Harper MJK, Bennett JP, Rowson LEA. Movement of the ovum in the reproductive tract. Nature 1961; 190:788.

14. Poindexter AN III, Thompson DJ, Gibbons WE, Findley WE, Dodson MG, Young RL. Residual embryos in failed embryo transfer. Fertil Steril 1986; 46:262.
15. Ménézo L, Anker D, Salat-Baroux J. Conception and realization of artificial dried embryo for training in IVF. Acta Eur Fertil 1985; 16:1.
16. Knutzen V, Stratton CJ, Sher G, McNamee PI, Huang TT, Soto-Albors C. Mock embryo transfer in early luteal phase, the cycle before in vitro fertilization and embryo transfer: a descriptive study. Fertil Steril 1992; 57:156.
17. Mansour RT, Aboulghar MA, Serour GI, Amin YM. Dummy embryo transfer using methylene blue dye. Hum Reprod 1994; 9:1257.
18. Schulman JD. Delayed expulsion of transfer fluid after IVF/ET. Lancet 1986; 1:44.
19. Woolcott R, Stanger J. Potentially important variables identified by transvaginal ultrasound-guided embryo transfer. Hum Reprod 1997; 12:963.
20. Fanchin R, Righini C, Olivennes F, Taylor S, de Ziegler D, Frydman R. Uterine contractions at the time of embryo transfer alter pregnancy rates after in vitro fertilization. Hum Reprod 1998; 13:1968.
21. Patton PE, Stoelk EM. Difficult embryo transfer managed with a coaxial catheter system. Fertil Steril 1993; 60:182.
22. Zreik TG, Dickey KW, Keefe DL, Glickman MG, Olive DL. Fluoroscopically guided cervical dilatation in patients with infertility. J Am Assoc Gynecol Laparosc 1996; 4(suppl 56):S56.
23. Garcia J. Conceptus transfer. In: Jones G, Jones G, Hodgen G, Rosen-Waks Z, eds. In Vitro Fertilization. Norfolk, Baltimore: Williams and Wilkins, 1986:215.
24. Nabi A, Awonuga A, Birch H, Barlow S, Stewart B. Multiple attempts at embryo transfer: does this affect in vitro fertilization treatment outcome? Hum Reprod 1997; 12:1188.
25. Egbase PE, Al-Sharhan M, Al-Othman S, Al-Mutawa M, Udo EE, Grudzinskas JG. Incidence of microbial growth from the tip of the embryo transfer catheter after embryo transfer in relation to clinical pregnancy rate following in vitro fertilization and embryo transfer. Hum Reprod 1996; 11:1687.
26. Fanchin R, Harmas A, Benaoudia F, Lundkvist U, Olivennes F, Frydman R. Microbial flora of the cervix assessed at the time of embryo transfer adversely affects in vitro fertilization outcome. Fertil Steril 1998; 70:866.
27. Mansour R, Aboulghar M, Serour G. Dummy embryo transfer: a technique that minimizes the problems of embryo transfer and improves the pregnancy rate in human in vitro fertilization. Fertil Steril 1990; 54:678.
28. Abusheikhha N, Lass A, Akagbosu F, Brinsden P. How useful is cervical dilation in patients with cervical stenosis who are participating in an in vitro fertilization–embryo transfer program? The Bourn Hall experience. Fertil Steril 1999; 72:610.
29. Prapas N, Prapas Y, Panagiotidis Y, Prapa S, Vanderzwalmen P, Makedos G. Cervical dilatation has a positive impact on the outcome of IVF in randomly assigned cases having two previous difficult embryo transfers. Hum Reprod 2004; 19:1791.
30. Glatstein IZ, Pang SC, McShane PM. Successful pregnancies with the use of laminaria tens before embryo transfer for refractory cervical stenosis. Fertil Steril 1997; 67:1172.

31. Shamonki MI, Spandorfer SD, Rosenwaks Z. Ultrasound-guided embryo transfer and the accuracy of trial embryo transfer. Hum Reprod 2005; 3:709.
32. Sallam HN, Agameya AF, Rahman AF, Ezzeldin F, Sallam AN. Ultrasound measurement of the uterocervical angle before embryo transfer: a prospective controlled study. Hum Reprod 2002; 7:1767.
33. Birnholz JC. Ultrasonic visualization of endometrial myometrial movements. Fertil Steril 1984; 41:157.
34. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Embryo transfer—can we learn anything new from the observation of junctional zone contractions? Hum Reprod 1998; 13:1540.
35. Kerin JF, Jeffrey R, Warnes GM, Cox LW, Broom TJ. A simple technique for human embryo transfer into the uterus. Lancet 1981; 2(8249):726.
36. Leeton J, Trounson A, Jessup D, Wood C. The technique for embryo transfer. Fertil Steril 1982; 14:2417.
37. Diedrich K, Van der Ven H, Al-Hasani S, Krebs D. Establishment of pregnancy related to embryo transfer techniques after in vitro fertilization. Hum Reprod 1989; 4:111.
38. Egbase PE, Al-Sharhan M, Grudzinskas JG. Influence of position and length of uterus on implantation and clinical pregnancy rates in IVF and embryo transfer treatment cycles. Hum Reprod 2000; 15:1943.
39. Al-Shawaf T, Dave R, Harper J, Linehan D, Riley P, Craft I. Transfer of embryos into the uterus: how much do technical factors affect pregnancy rates? J Assist Reprod Genet 1993; 10:31.
40. Waterstone J, Curson R, Parsono J. Embryo transfer to low uterine cavity. Lancet 1991; 337:1413.
41. Rosenlund B, Sjoblom P, Hillensjo T. Pregnancy outcome related to the site of embryo deposition in the uterus. J Ass Reprod Genet 1996; 13:511.
42. Naaktgeboren N, Broers FC, Heijnsbroek I. Hard to believe hardly discussed, nevertheless very important for the IVF/ICSI results: embryo transfer technique can double or halve the pregnancy rate. Hum Reprod 1997; 12:149.
43. Frankfurter D, Trimarchi JB, Silva CP, Keefe DL. Middle to lower uterine segment embryo transfer improves implantation and pregnancy rates compared with fundal embryo transfer. Fertil Steril 2004; 5:1273.
44. Oliveira JB, Martins AM, Baruffi RL, et al. Increased implantation and pregnancy rates obtained by placing the tip of the transfer catheter in the central area of the endometrial cavity. Reprod Biomed Online 2004; 9:435.
45. Coroleu B, Barri PN, Carreras O. The influence of the depth of embryo replacement into the uterine cavity on implantation rates after IVF: a controlled, ultrasound guided study. Hum Reprod 2002; 17:341.
46. van de Pas MM, Weima S, Looman CW, Broekmans FJ. The use of fixed distance embryo transfer after IVF/ICSI equalizes the success rates among physicians. Hum Reprod 2003; 4:774.
47. Fraser IS. Prostaglandins, prostaglandin inhibitors and their roles in gynaecological disorders, Baillieres. Clin Obstet Gynaecol 1992; 6:829.
48. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Embryo transfer and uterine junctional zone contractions. Hum Reprod Update 1999; 5:87.

49. Choe JK, Nazari A, Check JH, Summers-Chase D, Swenson K. Marked improvement in clinical pregnancy rates following in vitro fertilization–embryo transfer seen when transfer technique and catheter were changed. Clin Exp Obstet Gynecol 2001; 4:23.
50. Wood EG, Batzer FR, Go KJ, Gutmann JN, Corson SL. Ultrasound-guided soft catheter embryo transfers will improve pregnancy rates in in vitro fertilization. Hum Reprod 2000; 15:107.
51. Wisanto A, Janssens R, Deschacht J, Camus M, Devroey P, Van Steirteghem AC. Performance of different embryo transfer catheters in a human in vitro fertilization program. Fertil Steril 1989; 52:79.
52. Ghazzawi IM, Al-Hasani S, Karaki R, Souso S. Transfer technique and catheter choice influence the incidence of transcervical embryo expulsion and the outcome of IVF. Hum Reprod 1999; 14:677.
53. De Placido G, Wilding M, Stina I, et al. The effect of ease of transfer and type of catheter used on pregnancy and implantation rates in an IVF program. J Assist Reprod Genet 2002; 19:14.
54. Karande V, Hazlett D, Vietzke M, Gleicher N. A prospective randomized comparison of the Wallace catheter and the Cook Echo-Tip catheter for ultrasound-guided embryo transfer. Fertil Steril 2002; 4:826.
55. Burke LM, Davenport AT, Russell GB, Deaton JL. Predictors of success after embryo transfer: experience from a single provider. Am J Obstet Gynecol 2000; 5:1001.
56. van Weering HG, Schats R, McDonnell J, Vink JM, Vermeiden JP, Hompes PG. The impact of the embryo transfer catheter on the pregnancy rate in IVF. Hum Reprod 2002; 3:666.
57. McDonald JA, Norman RJ. A randomized controlled trial of a soft double lumen embryo transfer catheter versus a firm single lumen catheter: significant improvements in pregnancy rates. Hum Reprod 2002; 6:1502.
58. Penzias A, Harris D, Barrett C, et al. Outcome oriented research in an IVF program: transfer catheter type affects IVF outcome [abstr]. In: Proceedings of the 53rd Annual Meeting of the American Society for Reproductive Medicine, 1997:S163.
59. Abou-Setta AM, Al-Inany HG, Mansour RT, Serour GI, Aboulghar MA. Soft vs. firm embryo transfer catheters for assisted reproduction: a systematic review and meta-analysis. Hum Reprod 2005; 11:3114.
60. Dorn C, Reinsberg J, Schlebusch H, Prietl G, van der Ven H, Krebs D. Serum oxytocin concentration during embryo transfer procedure. Eur J Obstet Gynecol Reprod Biol 1999; 87:77.
61. Lesny P, Killick SR, Robinson J, Raven G, Maguiness SD. Junctional zone contractions and embryo transfer: is it safe to use a tenaculum? Hum Reprod 1999; 14:2367.
62. Englert Y, Puissant F, Camus M, Van Hoeck J, Leroy F. Clinical study on embryo transfer after human in vitro fertilization. J In Vitro Fert Embryo Transf 1986; 3:243.
63. Sharif K, Afnan M, Lenton W. Mock embryo transfer with a full-bladder immediately before the real transfer for in vitro fertilization treatment: the Birmingham experience of 113 cases. Hum Reprod 1995; 10:1715.

64. Tomas C, Tikkinen K, Tuomivaara L, Tapanainen JS, Martikainen H. The degree of difficulty of embryo transfer is an independent factor for predicting pregnancy. Hum Reprod 2002; 17:2632.
65. Alvero R, Hearns-Stokes RM, Catherino WH, Leondires MP, Segars JH. The presence of blood in the transfer catheter negatively influences outcome at embryo transfer. Hum Reprod 2003; 9:1848.
66. Murray AS, Healy DL, Rombauts L. Embryo transfer: hysteroscopic assessment of transfer catheter effects on the endometrium. Reprod Biomed Online 2003; 5:583.
67. Fanchin R, Righini C, de Ziegler D, Olivennes F, Ledee N, Frydman R. Effects of vaginal progesterone administration on uterine contractility at the time of embryo transfer. Fertil Steril 2001; 75:1136.
68. Ayoubi JM, Fanchin R, Kaddouz D, Frydman R, de Ziegler D. Uterorelaxing effects of vaginal progesterone: comparison of two methodologies for assessing uterine contraction frequency on ultrasound scans. Fertil Steril 2001; 76:736.
69. Baruffi R, Mauri AL, Petersen CG, Felipe V, Franco JG Jr. Effects of vaginal progesterone administration staring on the day of oocytes retrieval on pregnancy rates. J Assist Reprod Genent 2003; 20:517.
70. Dawood MY. Nonsteroidal anti-inflammatory drugs and reproduction. Am J Obstet Gynecol 1993; 169:1255.
71. Moon HS, Park SH, Lee JO, Kim KS, Joo BS. Treatment with piroxicam before embryo transfer increases the pregnancy rate after in vitro fertilization and embryo transfer. Fertil Steril 2004; 8:816.
72. Al-Inany HG, Wasseef M, Aboulghar MA, Mansour RT, Serour GI, Abou-Setta AM. Embryo transfer under propofol anesthesia: the impact on implantation and pregnancy rate. Middle East Fertil Soc J 2003; 8:269.
73. Neithardt AB, Segars JH, Hennessy S, James AN, Mckeeby JL. Embryo after loading: a refinement in embryo transfer technique that may increase clinical pregnancy. Fertil Steril 2005; 83:710.
74. Lewin A, Schenker JG, Avrech O, Shapira S, Safran A, Friedler S. The role of uterine straightening by passive bladder distension before embryo transfer in IVF cycles. J Assist Reprod Genet 1997; 14:32.
75. Sundstrom P, Wramsby H, Persson PH, Liedholm P. Filled bladder simplifies human embryo transfer. Br J Obstet Gynaecol 1984; 91:506.
76. Nielsen IK, Lindhard A, Loft A, Ziebe S, Andersen AN. A Wallace malleable stylette for difficult embryo transfer in an in vitro fertilization program: a case–control study. Acta Obstet Gynecol Scand 2002; 2:133.
77. Kruger TF, Stander FS, van der Merwe JP, Smith K, Menkveld R. The technique for human embryo transfer at Tygerberg Hospital. Acta Eur Fertil 1986; 2:117.
78. Johnson N, Bromham DR. Effect of cervical traction with a tenaculum on the uterocervical angle. Br J Obstet Gynaecol 1991; 98:309.
79. Silberstein T, Weitzen S, Frankfurter D, Trimarchi JR, Keefe DL, Plosker SM. Canulation of a resistant internal os with the malleable outer sheath of a coaxial soft embryo transfer catheter does not affect in vitro fertilization–embryo transfer outcome. Fertil Steril 2004; 5:1402.

80. Helsa J, Stevens J, Schlenker T. Comparison of malleable stylette Wallace catheter to Tomcat catheter for difficult embryo transfer [abstr]. Fertil Steril 1998; 70(suppl 1):S222.
81. Yanushpolsky EH, Ginsburg ES, Fox JH, Stewart EA. Transcervical placement of a Malecot catheter after hysteroscopic evaluation provides for easier entry into the endometrial cavity for women with histories of difficult intrauterine inseminations and/or embryo transfers: a prospective case series. Fertil Steril 2000; 73:402.
82. Groutz A, Lessing J, Wolf Y, Azem F, Yovel I, Amit A. Comparison of transmyometrial and transcervical embryo transfer in patients with previously failed in vitro fertilization–embryo transfer cycles and/or cervical stenosis. Fertil Steril 1997; 67:1073.
83. Serhal P, Ranieri DM, Khadum I, Wakim RA. Cervical dilatation with hygroscopic rods prior to ovarian stimulation facilitates embryo transfer. Hum Reprod 2003; 18:2618.
84. Wood C, McMaster R, Rennie G, Trounson A, Leeton J. Factors influencing pregnancy rates following in vitro fertilization and embryo transfer. Fertil Steril 1985; 2:245.
85. Parsons JH, Bolton VN, Wilson L, Campbell S. Pregnancies following in vitro fertilization and ultrasound-directed surgical embryo transfer by perurethral and transvaginal techniques. Fertil Steril 1987; 4:691.
86. Kato O, Takatsuka R, Asch RH. Transvaginal–transmyometrial embryo transfer: the Towako method: experience of 104 cases. Fertil Steril 1993; 59:51.
87. Craft I, McLeod F, Edmonds K. Human embryo transfer technique. Lancet 1981; 2:1104.
88. Lenz S, Leeton J. Evaluating the possibility of uterine transfer by ultrasonically guided transabdominal puncture. J In Vitro Fert Embryo Transf 1987; 1:18.
89. Wikland M, Hamberger L, Enk L, Nilsson L. Sonographic techniques in human in vitro fertilization programmes. Hum Reprod 1988; 3(suppl 2):65.
90. Sharif K, Afnan M, Lenton W, Bilalis D, Hunjan M, Khalaf Y. Transmyometrial embryo transfer following difficult immediate mock transcervical. Fertil Steril 1996; 65:1071.
91. McNamee P, Huang T, Carwile A. Significant increase in pregnancy rates achieved by vigorous irrigation of endocervical mucus prior to embryo transfer with a Wallace catheter in an IVF-ET program [abstr]. Fertil Steril 1998; 70(suppl 1):S228.
92. Sallam HN, Farrag A, Ezzeldin F, Agameya A, Sallam AN. The importance of flushing the cervical canal with cluture medium prior to embryo transfer. Fertil Steril 2000; 74(3)suppl 11:S64–S65.
93. Glass KB, Green CA, Fluker MR, Schoolcraft WB, McNamee PI, Meldrum D. Multicenter randomized trial of cervical irrigation at the time of embryo transfer [abstr]. Fertil Steril 2000; 74(suppl 1):S31.
94. Mansour R. Minimizing embryo expulsion after embryo transfer: a randomized controlled study. Hum Reprod 2005; 1:170.
95. Strickler RC, Christianson C, Crane JP. Ultrasound guidance for human embryo transfer. Fertil Steril 1985; 43:54.

96. Leong M, Leung C, Tucker M, Wong C, Chan H. Ultrasound-assisted embryo transfer. J In Vitro Fert Embryo Transf 1986; 3:383.
97. Hurley VA, Osborn JC, Leoni MA, Leeton J. Ultrasound-guided embryo transfer: a controlled trial. Fertil Steril 1991; 3:559.
98. Kan AK, Abdalla HI, Gafar AH, et al. Embryo transfer: ultrasound-guided versus clinical touch. Hum Reprod 1999; 14:1259.
99. Lindheim SR, Cohen MA, Sauer MV. Ultrasound guided embryo transfer significantly improves pregnancy rates in women undergoing oocyte donation. Int J Gynaecol Obstet 1999; 66:281.
100. Coroleu B, Carreras O, Veiga A, et al. Embryo transfer under ultrasound guidance improves pregnancy rates in in vitro fertilization. Hum Reprod 2000; 15:616.
101. Prapas Y, Prapas N, Hatziparasidou A, et al. Ultrasound-guided embryo transfer maximizes the IVF results on day 3 and day 4 embryo transfer but has no impact on day 5. Hum Reprod 2001; 9:1904.
102. Tang OS, Ng EH, So WW, Ho PC. Ultrasound guided embryo transfer: a prospective randomized controlled trial. Hum Reprod 2001; 11:2310.
103. Anderson RE, Nugent NL, Gregg AT, Nunn SL, Behr BR. Transvaginal ultrasound-guided embryo transfer improves outcome in patients with previous failed in vitro fertilization cycles. Fertil Steril 2002; 4:769.
104. Matorras R, Urquijo E, Mendoza R, Corcostegui B, Exposito A, Rodriguez-Excudero FJ. Ultrasound guided embryo transfer improves pregnancy rates and increase the frequency of easy transfers. Hum Reprod 2002; 17:1762.
105. Li R, Lu L, Hao G, Zhong K, Cai Z, Wang W. Abdominal US guided embryo transfer improves clinical pregnancy rates after in vitro fertilization: experiences from 330 clinical investigations. J Assist Reprod Genet 2005; 22:3.
106. Garcia-Velasco JA, Isaza V, Martinez-Salazar J, et al. Transabdominal ultrasound-guided embryo transfer does not increase pregnancy rates in oocyte recipients. Fertil Steril 2002; 3:534.
107. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. Fertil Steril 2003; 4:1037.
108. Sohan K, Woodward B, Ramsewak SS. Successful use of transrectal ultrasound for embryo transfer in obese women. J Obstet Gynaecol 2004; 24:839.
109. Jones HW Jr., Acosta AA, Garcia JE, Sandow BA, Veeck L. On the transfer of conceptuses from oocytes fertilized in vitro. Fertil Steril 1983; 2:241.
110. Yovich JL, Turner SR, Murphy AJ. Embryo transfer technique as a cause of ectopic pregnancies in in vitro fertilization. Fertil Steril 1985; 44:318.
111. Vaught LK, Stratton CJ, Julian P, et al. An improved system for catheter loading during embryo transfer. J In Vitro Fert Embryo Transf 1987; 6:346.
112. Kremple E, Zegermacher G, Eichler C, Obruca A, Strohmer H, Feichtinger W. Air in the uterine cavity after embryo transfer. Fertil Steril 1995; 2:366.
113. Moreno V, Balasch J, Vidal E, Calafell JM, Civico S, Vanrell JA. Air in the transfer catheter does not affect the success of embryo transfer. Fertil Steril 2004; 5:1366.
114. Ebner T, Yaman C, Moser M, Sommergruber M, Polz W, Tews G. The ineffective loading process of the embryo transfer catheter alters implantation and pregnancy rates. Fertil Steril 2001; 3:630.

115. Khan I, Staessen C, Devroey P, Van Steirteghem AC. Human serum albumin versus serum: a comparative study on embryo transfer medium. Fertil Steril 1991; 56:98.
116. Menezo Y, Arnal F, Humeau C, Ducret L, Nicollet B. Increased viscosity in transfer medium does not improve the pregnancy rates after embryo replacement. Fertil Steril 1989; 52:680.
117. Tur-Kaspa I, Yuval Y, Bider D, Levron J, Shulman A, Dor J. Difficult or repeated sequential embryo transfers do not adversely affect in vitro fertilization pregnancy rates or outcome. Hum Reprod 1998; 9:2452.
118. Goudas VT, Hammitt DG, Damario MA, Session DR, Singh AP, Dumesic DA. Blood on the embryo transfer catheter is associated with decreased rates of embryo implantation and clinical pregnancy with the use of in vitro fertilization–embryo transfer. Fertil Steril 1998; 70:878.
119. Awonuga A, Nabi A, Govindbhai J, Birch H, Stewart B. Contamination of embryo transfer catheter and treatment outcome in in vitro fertilization. J Ass Reprod Genet 1998; 4:198.
120. Leeton HC, Seifer DB, Shelden RM. Impact of retained embryos on the outcome of assisted reproductive technologies. Fertil Steril 2004; 2:334.
121. Edwards RG, Steptoe PC, Purdy JM. Establishing full-term human pregnancies using cleaving embryos grown in vitro. Br J Obstet Gynaecol 1980; 9:737.
122. Lopata A, Johnston IW, Hoult IJ, Speirs AI. Pregnancy following intrauterine implantation of an embryo obtained by in vitro fertilization of a preovulatory egg. Fertil Steril 1980; 2:117.
123. Damewood MD, Meng SG. Personal and the organization of the IVF team. In: Damewood MD, ed. The Johns Hopkins Handbook of In Vitro Fertilization and Assisted Reproductive Technologies. Boston: Little, Brown and Company 1990:39.
124. Botta G, Graudzinskas G. Is a prolonged bed rest following embryo transfer useful? Hum Reprod 1997; 12:2489.
125. Sherif K, Afnan M, Lashen H, et al. Is bed rest following embryo transfer necessary? Fertil Steril 1998; 69:478.
126. Woolcott R, Stanger J. Ultrasound tracking of the movement of embryo-associated air bubbles on standing after transfer. Hum Reprod 1998; 8:2107.
127. Bar-Háva I, Kerner R, Yoeli R, Ashknazi J, Shalev Y, Orvieto R. Immediate ambulation after embryo transfer: a prospective study. Fertil Steril 2005; 83:594.
128. de Vries K, Lyons EA, Ballard G, Levi CS, Lindsay DJ. Contractions of the inner third of the myometrium. Am J Obstet Gynecol 1990; 3:679.
129. Lindholm P, Sundstrom P, Wramsby H. A model for experimental studies on human egg transfer. Arch Androl 1980; 5:92.
130. Baba K, Ishihara O, Hayashi N, Saitoh M, Taya J, Kinoshita K. Where does the embryo implant after embryo transfer in humans? Fertil Steril 2000; 73:123.
131. Zeck H, Stecher A, Riedler L, et al. High implantation rate with a fast and atraumatic embryo transfer technique [abstr Bk 1]. Hum Reprod 1997; 12:156.
132. Martinez F, Coroleu B, Parriego M, et al. Ultrasound-guided embryo transfer: immediate withdrawal of the catheter versus a 30 second wait. Hum Reprod 2001; 5:871.

133. Devroey P, Bracckmans P, Smitz J, et al. Pregnancy after translaparoscopic zygote intrafallopian transfer in a patient with sperm antibodies. Lancet 1986; 1:1329.
134. Yovich JL, Blackledge DG, Richardson PA, Matson PL, Turner SR, Draper R. Pregnancies following pronuclear stage tubal transfer. Fertil Steril 1987; 5:851.
135. Balmaceda JP, Gastaldi C, Remohi J, Borrero C, Ord T, Asch RH. Tubal embryo transfer as a treatment for infertility due to male factor. Fertil Steril 1988; 3:476.
136. Abyholm T, Tanbo T, Henriksen T, Magnus O. Preliminary experience with embryo intrafallopian transfer (EIFT). Int J Fertil 1990; 6:339.
137. Risquez F, Boyer P, Rolet F, et al. Retrograde tubal transfer of human embryos. Hum Reprod 1990; 2:185.
138. Cohen J, Mayaux MJ, Guihard-Moscato ML, Schwartz D. In vitro fertilization and embryo transfer: a collaborative study of 1163 pregnancies on the incidence and risk factors of ectopic pregnancies. Hum Reprod 1986; 4:255.
139. Pope CS, Cook EK, Arny M, Novak A, Grow DR. Influence of embryo transfer depth on in vitro fertilization and embryo transfer outcomes. Fertil Steril 2004; 1:51–58.
140. Nazari A, Askari HA, Check JH, O'Shaughnessy A. Embryo transfer technique as a cause of ectopic pregnancy in in vitro fertilization. Fertil Steril 1993; 5:919.
141. Bennett S, Waterstone J, Parsons J, Creighton S. Two cases of cervical pregnancy following in vitro fertilization and embryo transfer to the lower uterine cavity. J Assist Reprod Genet 1993; 10:100.
142. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Uterine junctional zona contraction during assisted reproduction cycles. Hum Reprod Update 1998; 4:440.
143. Khalifa Y, Redgment CJ, Yazdani N, Taranissi M, Craft IL. Intramural pregnancy following difficult embryo transfer. Hum Reprod 1994; 9:2427.
144. Sallam H, Sadek A, Ezzeldin F. Does cervical infection affect the results of IVF and ICSI? A meta-analysis off controlled studies. Fertil Steril 2003; 80:S110.
145. Sauer MV, Paulson RJ. Pelvic abscess complicating transcervical embryo transfer. Am J Obstet Gynecol 1992; 166(1 Pt 1):148.
146. Robert G, Edwards. The beginnings of human in vitro fertilization. In: Garden D, Weissman A, Howles C, Shoham Z, eds. Assisted Reproductive Techniques: Laboratory and Clinical Perspectives. UK: Martin Dumitz, 2001:1.
147. Garcia J, Jones C, Acosta A, Wright G. Corpus luteum function after follicle aspiration for oocyte retrieval. Fertil Steril 1981; 36:565.
148. Vargyas JM, Kletzly OA, Marrs RT. The effect of laparoscopic follicular aspiration on ovarian steroidogenesis during the early preimplantation period. Fertil Steril 1986; 45:221.
149. Csapo AI, Pulkkinen MO, Wiest WG. Effects of luteectomy and progesterone replacement therapy in early pregnant patients. Am J Obstet Gynecol 1973; 115:759.
150. Csapo AI, Pulkkinen MO, Kaihola HL. The relationship between the timing of luteectomy and the incidence of complete abortions. Am J Obstet Gynecol 1974; 118:985.

151. de Mouzon, Lancaster PI. World Collaborative Report on In Vitro Fertilization—1993. XVth World Congress on Fertility and Sterility, Montpellier, September 17, 1995.
152. Hughes EG, Fedorkow DM, Daya S, Sagle MA, Van de Koppel P, Collins JA. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: a meta-analysis of randomized controlled trials. Fertil Steril 1992; 58:888.
153. Macklon NS, Fauser BC. Impact of ovarian hyperstimulation on the luteal phase. J Reprod Fertil Suppl 2000; 55:101.
154. Smitz J, Devroey P, Camus M, et al. The luteal phase and early pregnancy after combined GnRH-agonist/HMG treatment for superovulation in IVF or GIFT. Hum Reprod 1988; 3:585.
155. Smitz J, Erard P, Camus M, et al. Pituitary gonadotrophin secretory capacity during the luteal phase in superovulation using GnRH-agonists and HMG in a desensitization or flare-up protocol. Hum Reprod 1992; 7:1225.
156. Lin Y, Kahn JA, Hillensjo T. Is there a difference in the function of granulosa-luteal cells in patients undergoing in vitro fertilization either with gonadotrophin-releasing hormone agonist or gonadotrophin-releasing hormone antagonist? Hum Reprod 1999; 14:885.
157. Soliman S, Daya S, Collins J, Hughes EG. The role of luteal phase support in infertility treatment: a meta-analysis of randomized trials. Fertil Steril 1994; 61:1068.
158. Pritts EA, Atwood AK. Luteal phase support in infertility treatment: a meta-analysis of the randomized trials. Hum Reprod 2002; 17:2287.
159. Penzias AS. Luteal phase support. Fertil Steril 2002; 77:318.
160. Daya S, Gunby J. Luteal phase support in assisted reproduction cycles. Cochrane Database Syst Rev 2004; (3):CD004830.
161. Penzias AS, Alper MM. Luteal support with vaginal micronized progesterone gel in assisted reproduction. Reprod Biomed Online 2003; 6:287.
162. Smitz J, Devroey P, Faguer B, Bourgain C, Camus M, Van Steirteghem AC. A prospective randomized comparison of intramuscular or intravaginal natural progesterone as a luteal phase and early pregnancy supplement. Hum Reprod 1992; 7:168.
163. Artini PG, Volpe A, Angioni S, Galassi MC, Battaglia C, Genazzani AR. A comparative, randomized study of three different progesterone support of the luteal phase following IVF/ET program. J Endocrinol Invest 1995; 18:51.
164. Abate A, Perino M, Abate FG, Brigandi A, Costabile L, Manti F. Intramuscular versus vaginal administration of progesterone for luteal phase support after in vitro fertilization and embryo transfer: a comparative randomized study. Clin Exp Obstet Gynecol 1999; 26(3–4):203.
165. Anserini P, Costa M, Remorgida V, Sarli R, Guglielminetti E, Ragni N. Luteal phase support in assisted reproductive cycles using either vaginal (Crinone 8) or intramuscular (Prontogest) progesterone: results of a prospective randomized study. Minerva Ginecol 2001; 53:297.
166. Guesa S, Causio F, Marinaccio M, Stanzia A, Sarcina E. Luteal phase support with progesterone in IVF/ET cycles: a prospective, randomized study

comparing vaginal and intramusclar administration. In: Abstract of the 17th Annual Meeting of the ESHRE, Switzerland, Abstract, 2001:111.
167. Costabile L, Gerli S, Manna C, Rossetti D, Di Renzo GC, Unfer V. A prospective randomized study comparing intramuscular progesterone and 17alpha-hydroxyprogesterone caproate in patients undergoing in vitro fertilization–embryo transfer cycles. Fertil Steril 2001; 76:394.
168. Check JH, Nowroozi K, Chase J, Nazari A, Callan C. Comparison of luteal-phase support with high- and low-dose progesterone therapy on pregnancy rates in an in vitro fertilization program. J In Vitro Fert Embryo Transf 1991; 8:173.
169. Bouckaert Y, Robert F, Englert Y, De Backer D, De Vuyst P, Delbaere A. Acute eosinophilic pneumonia associated with intramuscular administration of progesterone as luteal phase support after IVF: case report. Hum Reprod 2004; 19:1806.
170. Friedler S, Raziel A, Schachter M, Strassburger D, Bukovsky I, Ron-El R. Luteal support with micronized progesterone following in vitro fertilization using a down-regulation protocol with gonadotrophin-releasing hormone agonist: a comparative study between vaginal and oral administration. Hum Reprod 1999; 14:1944.
171. Licciardi FL, Kwiatkowski A, Noyes NL, Berkeley AS, Krey LL, Grifo JA. Oral versus intramuscular progesterone for in vitro fertilization: a prospective randomized study. Fertil Steril 1999; 71:614.
172. Farhi J, Weissman A, Steinfeld Z, Shorer M, Nahum H, Levran D. Estradiol supplementation during the luteal phase may improve the pregnancy rate in patients undergoing in vitro fertilization–embryo transfer cycles. Fertil Steril 2000; 73:761.
173. Unfer V, Casini ML, Gerli S, Costabile L, Mignosa M, Di Renzo GC. Phytoestrogens may improve the pregnancy rate in in vitro fertilization–embryo transfer cycles: a prospective, controlled, randomized trial. Fertil Steril 2004; 82:1509.

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Monitoring In Vitro Fertilization Outcome

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INTRODUCTION

In this chapter, I will provide insights into the following areas of in vitro fertilization outcome. First, why we monitor in vitro fertilization (IVF) outcome. Second, why monitoring IVF outcome is not well done. Third, a brief overview of the known IVF literature. Fourth, how to do monitoring in an ideal world, and what outstanding questions have not been addressed which are of concern to families, fertility practitioners, the broader scientific community, and general public.

WHY MONITOR IVF OUTCOME?

The first generation of assisted reproductive technology (ART)-conceived children are now growing up and ART practice has changed much during this period. The initial method of IVF has been supplemented by embryo cryopreservation and more recently by intracytoplasmic sperm injection (ICSI). Following on from these procedures, trans epididimal sperm aspiration (TESA) and testicular biopsy have resulted in a less naturally selective form of reproduction. These developments and also such things as extended culture, blastocyst transfers, etc., are often used without explicit

consideration of the risks for the child. However, several positive practice developments are underway now, including a genuine effort (underpinned by legislation) to reduce the risk of higher-order births (still the main risk to children born after ART), and efforts to consider the well-being of the child more formally from the start of new therapies. For example, I have been involved recently as an advisor on a (confidential) trial which investigates the efficacy of a treatment to enhance embryo implantation. The study designers, from the outset, asked advice on how to assess the health of any children born after successful pregnancies, both at birth and one year, with possibly longer-term plans.

Subfertile parents who conceived by IVF in its various forms are per se a skewed population of individuals whose offspring may well be at risk of problems via their parents' genetic natures, rather than the procedural-based aspects of their treatment for subfertility. This is not to trivialize the treatment-related aspects that are a topic of some concern, particularly at the present time when animal experiments seem to be backed up by recent human-related literature that suggests, e.g., imprinting disorders are sometimes a higher risk for children conceived after assisted reproductive therapies (see below). Families who are going to have assisted conception in its various forms, will often ask what the risks are to potential offspring as well as regarding the more immediate risk to themselves as patients undergoing what are often invasive and unpleasant procedures in order to conceive.

Crude epidemiological data have shown some striking phenomena as a result of ART, the most obvious of those is the birth of higher-order birth children such as triplets or quadruplets. These children themselves are at risk of problems as a direct consequence of having to share the fetal environment with their siblings and being born early.

Whenever a new form of ART is introduced (it seems that we are constantly, with the innovative nature of the development of fertility treatments, using seemingly more and more invasive methods of helping couples to conceive), there should be monitoring in place to look at the consequences for any births and pregnancies. Systematic follow-up is always better and if the processes are not in place to do that from the beginning, then attempting to "pick up the pieces" often leads to erroneous interpretation. A good example of monitoring resulting in changing a practice in the literature was the first child or groups of children born after round spermatid conception (1). Fortunately, these cases were reported, and as they had congenital anomalies, the procedure was suddenly banned by the Human Fertilization and Embryology Authority. Another example concerns the work of Eppig and O'Brien (2) in the United States, whose laboratory managed to persuade the primordial follicle of a mouse to be progressed through developmental stages to a mature follicle that then was inseminated, and subsequently the mouse which he called Eggbert was born. This mouse had an allegedly happy life but unfortunately developed a midlife crisis and dropped dead from obesity, diabetes, and sarcoma. Had this mouse's growth not been monitored,

it would have been unhelpful to the scientific community to not be aware that this mouse developed problems (albeit as an adult).

The message from this anecdote/case report is clear: the monitoring of children born after assisted conception must continue until they grow up. This is a sensitive and personal matter for the parents who must be considered in the conflict of interest of necessity versus privacy. Parents who are approached to participate in studies do not have to participate, but they must be made to see that it is useful to them as well as to the broader scientific and public communities. My assessments performed with these families have always given added value to those couples, families, and children.

WHAT ARE THE PROBLEMS KNOWN TO HAVE OCCURRED WITH OUTCOME STUDIES?

The first-choice approach in doing outcome studies concerning IVF is not to do them. This is the usual approach by most clinics throughout the world. I am only aware of two systematic follow-up clinics arranged specifically to monitor the outcome of ART: one is based in Sydney and is a local small affair, the other is with my collaborators in Belgium and arises from a very large clinic set up by former pediatrician Andre van Steirteghem. It is to his credit that he could see the necessity/appropriateness for such work. Other projects have been piecemeal or ad hoc. Common criticisms of the designs of studies looking at pediatric outcome include the following:

1. Poor matching criteria (3)
2. No controls (4)
3. Inappropriate tools of assessment (5)
4. Comparing two groups who superficially seem similar but are actually quite distinct (6)
5. Underpowered studies involving small numbers of children (4)
6. Mixing up groups of children, e.g., twins versus singletons versus triplets (7)
7. Poor response rates to follow-up (8)
8. Using multiple observers (9)
9. No blinding to conception status (10)

The ideal study has yet to be done and none of these cited studies were so, however, all contributed significantly to the understanding of ART outcome at the time of their publication.

WHY MONITOR: WHAT CAN GO WRONG IF THE DATA ARE MISUNDERSTOOD OR MISINTERPRETED?

An anecdote from my own clinical institution illustrates this well. A single woman was expecting a baby conceived with donor sperm and with the use of ICSI. The obstetrician had read that there was a higher risk of sex

chromosomal aneuploidy after ICSI and suggested that an amniocentesis for fetal karyotyping be performed. The literature underpinning this advice is weak and far from sufficient to give this advice to a pregnant woman. She refused the amniocentesis, but then became concerned. After birth, she requested a karyotype on her child and this was done. The karyotype was XYY. Subsequently, this nice little boy (who is now 8 years) is living with this "label," with the majority of the literature on this topic suggesting most children with this "variant" are normal and indeed grow up to be healthy adults. It could be argued that this bad advice and indeed the subsequent testing contravened the well-established guidelines concerning the clinical testing of children for genetic conditions. These guidelines should be rigidly applied and clearly state that where a condition has no health implications during childhood or an intervention cannot ameliorate the condition, there is no ethically justifiable reason for the child to be tested. Indeed, this child may well have refused such a test when grown up. I am sure the reader does not need to be convinced that the whole scenario would not have arisen if the child was not ART-conceived, and the Bonduelle et al. (11) work on the subject of aneuploidy after ICSI had been carefully checked.

WHAT IS KNOWN FROM THE CURRENT LITERATURE AS AN ALTERNATIVE?

Perinatal and Congenital Anomaly Studies of Children Conceived After ART

Herein lies the largest short-term risk for children born after ART, largely but not entirely due to the risk of higher-order births, well described after all types of ART. In developed nations, the rate of twinning has doubled in the past 25 years. This is thought to be 90% due to ART and 10% due to rising maternal age at first pregnancy. Fifty percent of twins are born at less than 2500 gms and 50% are born at less than 38 weeks gestation. Yet the risk of higher-order births (multiple pregnancies) after ART is 20% to 30%. There is one clinic in the United States where the service is no pregnancy—no fee, but that fee is alleged to be $20,000. The clinic replaces large numbers of embryos, despite the evidence that beyond two embryos, the only risk of three-embryo replacement is a triplet birth, not a higher overall pregnancy rate. To a pediatrician, this is grossly irresponsible. However, recently published guidelines in the United States (12), which are consistent with those in other countries, may well impact on such a practice.

What is the overall message from the literature concerning the risk of congenital anomalies and ART? Although one major study suggested a higher risk (doubling) of anomalies after ICSI generally, other large studies suggest that while there is a broadly increased risk of anomalies post-ART, that risk is modest. A large prospective study is needed and such a study has not been performed. Such studies are very expensive and all

studies to date can be criticized due to the inescapable fact that there may be experimental bias.

It is unsurprising that such a small increased risk of anomalies exists in view of the nature of the ART couple. Genetic factors come to bear in all types of infertility. With the advent of ICSI couples where there is predominantly male factor subfertility being able to reproduce, some clearly have known genetic defects resulting in the non-obstructive oligozoospermia that underlies the need for ICSI. Then there are other possible factors that may increase the risks from ART, such as culture media which may be relevant especially to the recently described increased risk of genomically imprintable disorders after ART, such as Beckwith Weidemann syndrome.

A summary of the three most major studies of congenital anomalies after different types of ART is provided in Table 1. Also, further commentary allows consideration of studies in different categories.

EVALUATION OF THE MAJOR PUBLISHED STUDIES

IVF Compared to the General Population

Different studies based on registry data have yielded contradictory results. After allowance for confounders, the difference in studies as authored by Westergaard et al. disappeared, but those by Hansen et al., even after adjusting for confounders such as maternal age, parity, and sex, still showed an increased risk of odds ratio (OR) of two. However, the Hansen et al. (6) study did not control for a number of variables, which could have been different in the two populations and could have led to different results.

ICSI Compared to the General Population

Retrospective Studies

In the Australian study (Hansen et al.) concerning congenital malformations at the age of a year, the OR remained two after adjustments. However, there was no allowance for years of infertility or socio-demographic factors, such as ethnic background, which may have been different in the two populations. Two Swedish analyses showed an increase in congenital malformations in ICSI and IVF; however, the adjustments for maternal age and other adjustments resulted in the differences disappearing.

Prospective Studies

There is only one prospective study, which was an excellent one, by a German group, Katalinic et al. (13). ICSI children ($n = 3372$) were compared to a select control group ($n = 8016$). This prospective study compared major malformations in ICSI and the naturally conceived (NC) population base. Here the risk, as stated in my summary above, was slightly above the natural population, at 1.24.

Table 1 Major Malformation Studies Few of Their Conclusion.

Authors	Study group	Study type	Outcome	Comments
Bonduelle	The follow-up study included agreement to genetic counseling and eventual prenatal diagnosis, followed by a physical examination of the children after 2 mo, after 1 yr, and after 2 yrs; 2840 ICSI children (1991–1999) and 2955 IVF children (1983–1999) were live born after replacement of fresh embryos; ICSI was carried out using ejaculated, epididymal, or testicular sperm	Ongoing retrospective cohort study	Major malformations (defined as those causing functional impairment or requiring surgical correction), were observed at birth in 3.4% of the ICSI live born children and in 3.8% of the IVF children ($P = 0.538$); malformation rate in ICSI was not related to sperm origin or sperm quality; the number of stillbirths (born ≥20 wks of pregnancy) was 1.69% in the ICSI group and 1.31% in the IVF group; total malformation rate taking into account major malformations in stillborns, in terminations, and in live borns was 4.2% in ICSI and 4.6% in IVF ($P = 0.482$)	A superb series of papers on this cohort hindered only by the comparison group being IVF and not ICSI

Katalinic	Three thousand three hundred and seventy-two children and fetuses and 8016 children and fetuses after the 16th wk of gestation in pregnancies after ICSI and natural conception, respectively	Prospective controlled study	The major malformation rate was 8.7% (295 of 3372) for the ICSI cohort and 6.1% (488 of 8016) for the population-based control cohort [relative risk, 1.44 (1.25–1.65)]; after adjustment for risk factors, the risk declined [adjusted odds ratio (OR), 1.24 (95% CI, 1.02–1.50)]; regarding singletons, there was a significant difference for birth weight and gestational age, with a higher number of preterm and low birth weight children in pregnancies achieved after ICSI substantially by the IVF centers in Germany themselves	In my view, the best study so far due to its prospective nature
Bergh	The medical records were retrieved for 1139 infants, 736 singletons, 200 sets of twins, and one set of triplets; the	Retrospective case–control study in which a smaller number of infants was compared	For ICSI children, the odd ratio (OR) for having any major or minor malformation was 1.75	Good-quality retrospective data compared to an acknowledged high-

(*Continued*)

Table 1 Major Malformation Studies Few of Their Conclusion. (*Continued*)

Authors	Study group	Study type	Outcome	Comments
	total number of infants with an identified anomaly was 87 (7.6%), 40 of which were minor; the incidence of malformations in children born after ICSI was also compared with all births in Sweden using data from the Swedish Medical Birth Registry and the Registry of Congenital Malformations	with the Swedish Medical Birth Registry	[95% confidence interval (CI) 1.19–2.58] after stratification for delivery hospital, year of birth, and maternal age; if stratification for singletons/twins was also done, the OR was reduced to 1.19 (95% CI 0.79–1.81); the increased rate of congenital malformations is thus mainly a result of a high rate of multiple births; the only specific malformation which was found to occur in excess in children born after ICSI was hypospadias (relative risk 3.0, exact 95% CI 1. 09–6.50) which may be related to paternal subfertility	quality national registry; disadvantage could be experimental bias in the index cases

Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

ICSI compared to the IVF

Bonduelle et al.'s excellent series of papers (8,14–16,19) gives valid comments on this topic and no difference in malformation rates has been found between ICSI and IVF children, the largest cohort being 2995 IVF versus 2899 ICSI.

Malformations in Different Organ Systems

None of the studies is sufficiently substantive to comment on this; however, there is emerging evidence from Bonduelle et al.'s work, and others suggest that urogenital malformations in ICSI are more common (17). This is surely unsurprising in view of the parental genetic background and the increased risk of male subfertility, when there are genitourinary defects in the father. Various sub-analyses have been performed to look at whether sperm quality and sperm source are relevant, but no clear message is available for this because of the limited number of children in sub-groups.

Developmental Outcome Studies of IVF and ICSI Children

Refer to Table 2 for an overview of some earlier IVF studies. Table 3 focuses on ICSI children. International collaborative study of ICSI–Child and family outcomes (ICSI–CFO), an international collaborative study of ICSI-child and family outcomes, is by far the largest (and most recent) study of IVF/ICSI children. It was performed in five European countries. Approximately 500 singleton ICSI, 500 IVF, and 500 NC children aged five years were each assessed with observer blinding to conception status. Confounders were avoided by ensuring that all children were more than 32 weeks gestation, singleton, matched for sex, social class, and Caucasian. These children were comprehensively assessed. This study showed no effect whatsoever of conception status on neurodevelopment (18), and although there was greater use of health service resources by ICSI and IVF children in relation to NC children, when examined in a comprehensive manner "top to toe," these children were not found to be physically different from NC children with the exception of congenital anomalies.

Developmental differences in an ICSI-conceived group of children when compared to conventional IVF and NC controls were reported in 1998 (19). The study found an increase in mild developmental delay using the Bayley scales of infant development to derive a mean developmental index (MDI). However, the study used comparison groups of IVF and NC children who were already enrolled in a separate study and had differing demographics to the ICSI group. There was also no blinding of the assessors and the number of participants in the study was small, with 89 ICSI-conceived children.

Bonduelle et al. have published several papers investigating congenital malformation rates and physical development of ICSI children (8,9,16,19–21).

Table 2 Developmental Outcome Studies for Conventional In Vitro Fertilization Children

Authors	Study group	Study type	Outcome	Key results	Comments
D'Souza et al. (1997)	278 IVF and 278 NC UK children; IVF singletons mean 25.5 mo (SD 7.9); IVF multiple births mean 24.8 mo (SD 5.1)	Prospective case–control study; matched for sex and social class	Results of Griffiths scales of development	Mean DQ: IVF singletons 116.9 (SD 12.6); IVF multiple births 106.9 (SD 10.9); not stated for controls; developmental delay (DQ < 70) noted in two multiple birth IVF children only	46% IVF children from multiple births; all controls were singleton; no matching for prematurity, birth weight or gestation
Cederblad et al. (1996)	99 Swedish IVF children (age 33–85 mo)	Single cohort compared to Swedish and American norms	Results of Griffiths scales of development	DQ above Swedish norm	No matched control group; high numbers of multiple births and prematurity
Brandes et al. (1992)	116 Israeli (Hebrew speaking) IVF children and 116 matched non-IVF children (age 12–45 mo)	Case–control study; matched for birth weight, gestational age, birth order, order in multiple births, mode of delivery, sex, age, maternal age, and education	Bayley scales for infants up to 30 mo; Stanford–Binet scales for children >30 mo; scales mean 100 ± 16	MDI Bayley scores: IVF 106 ± 19.6; non-IVF 110.6 ± 19.3; composite index for Stanford–Binet IVF 106.2 ± 8; non-IVF 104.4 ± 10.2	No correction for prematurity because children all >12 mo

Morin et al. (1989)	83 IVF children from Norfolk, Virginia, U.S.A. and 93 matched non-IVF children (age 12–30 mo)	Case–control study; matched for age, sex, race, multiple births, and maternal age	Results of Bayley scales: MDI and PDI; mean score 100	MDI scores: IVF 115 ± 13 ; non-IVF 111 ± 13 ; PDI scores: IVF 114 ± 14 ; non-IVF 108 ± 15	Study had power of 99% to detect difference; strongly suggests no difference; however, scores corrected for prematurity
Mushin et al. (1986)	33 Australian children (age 12–37 mo)	Single cohort from first 52 infants conceived at Monash IVF center; no matched controls	Results of Bayley scales; one child (37 mo) assessed using McCarthy scales	Overall MDI of 111 (SD = 15) and PDI of 105 (SD = 23); four children with physical and developmental problems had lower scores	High numbers of multiple births and prematurity; of four children with poor scores: two were VLBW, one severe CHD
Yovich et al. (1986)	20 Australian children (age 12–13 mo)	Single cohort of first 20 infants conceived after IVF in Western Australia	Results of Griffiths scales of development	General developmental quotient (DQ) was greater than mean of 100 in 19/20 children after correction for gestational age	No matched control group; increased rate of multiple births, IUGR, prematurity, and caesarean section

Abbreviations: DQ, development quotient; CHD, congenital heart disease; IUGR, intra uterine growth retardation; IVF, in vitro fertilization; MDI, mean developmental index; PDI, physical developmental index; VLBW, very low birth weight.
Source: Modified from unpublished thesis of Peters et al.

Table 3 Developmental Outcome Studies for Intracytoplasmic Sperm Injection Children

Authors	Study group	Study type	Outcome	Key results	Comments
Bonduelle, Wennerholm Loft, Tarlatzis, and Sutcliffe et al.	1515 children, 538 natural (NC); 437 IVF, 540 ICSI—Aged 5 yrs	Population control study Singleton, > 32 wks, Caucasian	Results of WPPSI, McCarthy Motor scales, Laterality Full physical check Growth, Audiometry Ophthalmic checks	Normal IQ; Normal laterality; Normal motor skills; Taller than NC peers; Higher anomalies	The most important study in the ART medical literature. In press. Ability at five is predictive of ability in adult life.
Sutcliffe et al. (2001)	208 UK children conceived after ICSI compared with 221 NC controls. Age 12–24 mo.	Case-control study. Matched for social class, maternal educational level, region, sex, and race.	Results of Griffiths scales of infant development	Griffiths quotients: ICSI 98.08 (SD 10.93); Controls 98.69 (SD 9.99)	No correction for gestational age in Griffiths scales. Single observer. 90% follow-up.
Bowen et al. (1998)	89 Australian ICSI children compared with 84 conventional IVF children and 80 NC. Assessed at birth and at	Prospective case-control study. Matched for parental age, parity, and	Results of Bayley scales of infant development	98% follow-up at 1 yr. MDI Bayley scores: ICSI 95.9 (SD 10.7)IVF	Included frozen embryos (39% ICSI, 31% IVF). Lack of blinding and differences in

	corrected age of 12 mo.	multiplicity of the pregnancy. Conventional and IVF children were recruited through separate study		101.8 (SD 8.5); Non-IVF 102.5 (SD 7.6)	sociodemographic factors, particularly between the parents of the ICSI group and other groups
Bonduelle et al. (1998)	201 Belgian (Dutch speaking) ICSI children compared with 131 conventional IVF children. Assessment age 22–26 mos	Blinded prospective case-control trial	Results of Bayley scales. Test results scored by subtracting chronological age from test age. Test age calculated from subset of 1283 Dutch children aged 2–30 mo	Scored mean age differences: ICSI singleton +2.11 (SD 3.12); IVF singleton +2.30 (SD 2.63); ICSI twin +1.67 (SD 3.06); IVF twin +0.31 (SD 3.75). Lower scores for triplets with males scoring lower than females	No correction for gestational age. Higher scores for singletons. Matching not discussed in this letter. Single observer. 60% follow-up

Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; MDI, mean developmental index; WPPSI, .
Source: Modified from unpublished thesis of Peters et al.

Several of these papers allude to the fact that developmental milestones were assessed, and formal assessments of these children, undertaken between 1995 and 1998, were published in a research letter to the Lancet in 1998 (18). This article reported 201 ICSI children and 131 IVF children who were assessed using Bayley, and the results were compared to a subset of children representing the Dutch population. The age of the children was not corrected for gestational age, but the ICSI and IVF children were found to have similar scores to the general population. The twins scored slightly lower than the singletons.

Sutcliffe et al. studied 208 singleton ICSI-conceived children at around 18 months and compared them with a matched NC control group (10,22). The children were assessed by a single observer using the Griffiths scales of mental development. No differences in developmental outcome were found between the two groups.

PHYSICAL ASSESSMENTS OTHER THAN FOR CONGENITAL ANOMALIES

Use of Medical Services

IVF children are more likely to need neonatal care, primarily because of the prematurity related to multiple pregnancies. Initial reports suggested that IVF children did not require extra medical attention after the neonatal period (23,24). Leslie studied 95 IVF children and compared them with 79 NC children matched for maternal age and parity. IVF children were also less likely to be breastfed by the time of discharge. However ICSI–CFO has disagreed with these findings and clearly shown greater use of medical resources among IVF/ICSI children, including surgery.

The point to emphasize here is that the ICSI–CFO study was performed with older children and was 10× as large as any of these early studies and thus was far more likely to detect a difference.

Growth

Saunders et al. published a case-matched control study of children conceived after assisted reproduction and found that the physical outcomes weight, head circumference, and malformation rates were no different between groups (24). The IVF group had a greater mean length percentile and the twins in each group had poorer physical outcomes, with an increase in prematurity and lower birth weights, and reduced height and weight at age two when compared to singletons in each group. Here the ICSI–CFO study concurs in showing that the growth standard deviation scores (SDS) scores for both IVF and ICSI are higher than for NC. This latter finding needs verification but is somewhat alarming, as so far nobody has accurately

charted growth of ART children, and so far larger children may have been "buried" in the effects of prematurity and higher-order births.

Retinopathy of Prematurity

The increase in multiple births and premature births related to assisted conception has led to an increase in conditions such as retinopathy that are directly related to early birth and low birth weight (25,26).

Anteby et al. reported the ocular manifestations in children born after IVF and referred for ophthalmological assessment. Major ocular malformations were found in 12 (26%) of the small cohort of 47 children studied (27). Seven major malformations were listed, including congenital cataract, optic atrophy, and retinoblastoma. The study was limited in power due to the small numbers of children involved and, because the study was conducted in a tertiary hospital, it is possible that the numbers were skewed due to the type of patients referred.

Childhood Cancer

There have been case reports of children conceived after assisted conception developing neuroectodermal tumors (28,29), but no large study has confirmed this finding. Bruinsma et al. used a record-linkage cohort design to link assisted reproduction births to a population-based cancer registry in Australia (30). This study included 5249 births and found no increase in the incidence of cancers in the assisted reproduction groups. However, these groups were relatively small and underpowered for the outcomes measured. The mean length of follow up was only three years nine months, although neuroblastomas tend to occur within the first year of life. These findings were supported by a smaller, similar Israeli study (31).

More recently, Klip et al. examined a large population-based historical cohort, established to investigate gynecological disorders in women undergoing IVF (32). This cohort included 9484 children whose mothers had been given IVF or related fertility treatments and 7532 children whose mothers were subfertile, but had conceived naturally. The mothers were mailed questionnaires enquiring about cancer in their children. There was a 67% response rate and no difference between the groups was noted, implying that IVF and related treatments do not increase the cancer risk to the child.

The cancer incidence in IVF children studied for the UK Medical Research Council (MRC) working party (33) and a Swedish national cohort study of IVF children (34) also found no increase in cancer rates, but the power of these studies was limited by a too small number of children studied. Doyle estimated that 20,000 children would be required to observe a doubling or halving of the risk of childhood cancer in children conceived after assisted reproduction compared with the general population. This would provide 95% significance and 90% power if children were followed up for four years (33).

Neurological Outcomes

There has been some suggestion from a Swedish study that children born after IVF have an increased risk of developing neurological problems, particularly cerebral palsy (35). They found a fourfold increase in risk of cerebral palsy in children born after IVF compared with matched controls—OR 3.7 (95% CI 2.0–6.6). The risk in singletons was nearly three times—OR 2.8 (95% CI 1.3–5.8). After adjusting for birth weight and a gestation of more than 37 weeks, the risk remained with an OR of 2.5 (95% CI 1.1–5.2). The authors admitted that the frequency of cerebral palsy in controls was lower than the Swedish norm. Calculations using their data indicate a prevalence of cerebral palsy in the control group as 1.5/1000 compared with an accepted prevalence rate of 2.0–2.5/1000 (36). The increased risk was shown to be mainly with multiple births and was associated with low birth weight and low gestational age. Leviton et al. noted that there is some over aggregation of the data, with children less than 30 weeks gestation grouped together. This does not allow for the effect of decreasing risk of cerebral palsy with increasing gestation, particularly in those infants born after 30 weeks (37). Also in a commentary by Sutcliffe, it was noted that the study used proxy measures for disability and that it was unexplained why the rate of problems seemed higher in the singleton group, than the IVF group, in contradiction to the entire twin literature!

GENOMIC IMPRINTING—THE ARTID STUDY

Assisted reproduction therapies and imprintable disorders (ARTID) is a UK survey (38). In the ARTID study, four conditions known to be imprintable in man were surveyed [Beckwith Weideman syndrome (BWS), Prader Willi syndrome (PWS)], Angelman syndrome (AS), and transient neonatal diabetes (TNDM). We confirmed an association between ART and BWS, and in particular, BWS caused by loss of maternal allele methylation at the KvDMR1 ICR. Although the absolute frequency of BWS after ART is small (<1%), this association is important as it confirms that in humans, as in some animals, ART may cause epigenetic changes that can lead to human disease. A key question is whether such epigenetic changes are restricted to BWS or may be associated with other phenotypes. Epimutations at the SNRPN ICR are a rare cause of AS (population frequency ~1 in 300,000) but to date five of seven children with AS conceived by ICSI have had this subtype (including our case) so it appears that ART-related BWS and AS may be specifically associated with maternal allele ICR methylation loss. The cause of the association between ART and loss of maternal allele ICR methylation in humans is uncertain, but two hypotheses have the most credence (39). Based on animal studies, it has been suggested in vitro embryo culture might predispose to KvDMR1 or SNRPN DMR LOM. Alternatively, there may be an increased

risk of an imprinting disorder following ART because of an association with infertility per se rather than with in vitro embryo culture (e.g., treatment for infertility such as ovarian hyperstimulation might be implicated and/or susceptibility to epigenetic defects might be responsible for both infertility and an increased risk of imprinting defects). To our knowledge, this is the first investigation of PWS and TNDM and ART. We did not find evidence of an association between ART and PWS and note that while paternal allele deletion and maternal uniparental disomy are the most common causes of PWS, methylation defects are rare and do not involve loss of methylation. In contrast, a subset of TNDM patients (~25%) has an isolated methylation defect (loss of maternal allele methylation) of an imprinted CpG island at chromosome 6q24. Although we did not find an increased frequency of ART in TNDM, this is a rare disorder and was the smallest patient group available for study.

Our study illustrates the problems encountered in undertaking research on possible long-term morbidity of ART without linkage between the HFEA database and the disease registers. Careful follow-up of ART children is required to define the precise risk of imprinting disorders and confirm or refute suggestions of risks of childhood tumors such as retinoblastoma (or others in which aberrant imprinting is part of the etiology). Our findings suggest that particular attention should be paid to disorders resulting from loss of DNA methylation in gene regulatory elements.

The clinician in fertility medicine should counsel his subfertile couple about the following risks from ART:

1. The highest risks are from prematurity (mainly from twins and higher-order births); therefore, single embryo replacement, at least in the first cycle, is recommended.
2. Mature babies are healthy generally and not at long-term health risk as a result of their mode of conception.
3. There is probably a higher risk of congenital anomalies after ART, which is at most double that of the general population (i.e., still a small risk).
4. It may be that in ICSI children there is specifically a higher risk of genitary urinary (GU) anomalies, again only a little above that for the NC population.
5. It is not possible to be sure that there are no longer-term risks from ART, as there are few children who have grown up and families are not always willing to agree to follow-up studies.
6. Monitoring is important, difficult to do and expensive, but is an essential part of assessing new advances in treatment after ART.

CONCLUSION

Generally, ART-conceived children who are born singleton and at term are similar in most longer-term outcomes to NC children (with the exception of

congenital anomalies). They do, however, appear to use more health service resources. There are questions which are unresolved concerning their progress into adult life.

These are as follows:

1. Are there longer-term risks of imprintable disorders and cancer?
2. Will these children be fertile when they are sexually mature?

ART-conceived children will be a significant client group as they grow up (at least 1% of the population in rich countries). If their ART conception has exposed them to undue risk because these factors were not studied when the techniques were introduced, they may well take a very different view of the justifications for ART than the readers of this chapter. Further studies need to be performed. The ideal one has yet to be done.

FURTHER READING

Sutcliffe AG. IVF Children the First Generation. Parthenon, 2002.

REFERENCES

1. Aslam I, Fishel S, Green S, Campbell A, Garratt L, McDermott H, et al. Can we justify spermatid microinjection for severe male factor infertility? Hum Reprod Update 1998; 4(3):213–222.
2. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 1996; 54(1):197–207.
3. Bowen JR, Gibson FL, Leslie GI, Saunders DM. Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. Lancet 1998; 351(9115):1529–1534.
4. Mushin DN, Barreda-Hanson MC, Spensley JC. In vitro fertilization children: early psychosocial development. J In Vitro Fert Embryo Transf 1986; 3(4):247–252.
5. Strom CM, Levin R, Strom S, Masciangelo C, Kuliev A, Verlinsky Y. Neonatal outcome of preimplantation genetic diagnosis by polar body removal: the first 109 infants. Pediatrics 2000; 106(4):650–653.
6. Hansen M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med 2002; 346(10):725–730.
7. Sutcliffe AG, D'Souza SW, Cadman J, Richards B, McKinlay IA, Lieberman B. Outcome in children from cryopreserved embryos. Arch Dis Child 1995; 72(4):290–293.
8. Bonduelle M, Joris H, Hofmans K, Liebaers I, Van Steirteghem A. Mental development of 201 ICSI children at 2 years of age. Lancet North Am Ed 1998; 351(9115):1553.
9. Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P, et al. Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999). Hum Reprod 2002; 17(3):671–694.

10. Sutcliffe AG, Taylor B, Li J, Thornton S, Grudzinskas JG, Lieberman BA. Children born after intracytoplasmic sperm injection: population control study. BMJ 1999; 318(7185):704–705.
11. Bonduelle M, Aytoz A, Van Assche E, Devroey P, Liebaers I, Van Steirteghem A. Incidence of chromosomal aberrations in children born after assisted reproduction through intracytoplasmic sperm injection. Hum Reprod 1998; 13(4):781–782.
12. Rebar RW, DeCherney AH. Assisted reproductive technology in the United States. N Engl J Med 2004; 350(16):1603–1604.
13. Katalinic A, Rosch C, Ludwig M. Pregnancy course and outcome after intracytoplasmic sperm injection (ICSI)—a controlled, prospective cohort study. Fertil Steril. In press.
14. Bonduelle M, Desmyttere S, Buysse A, et al. Prospective follow-up study of 55 children born after subzonal insemination and intracytoplasmic sperm injection. Hum Reprod 1994; 9(9):1765–1769.
15. Bonduelle M, Legein J, Derde MP, et al. Comparative follow-up study of 130 children born after intracytoplasmic sperm injection and 130 children born after in vitro fertilization. Hum Reprod 1995; 10 (12):3327–3331.
16. Bonduelle M, Legein J, Buysse A, et al. Prospective follow-up study of 423 children born after intracytoplasmic sperm injection. Hum Reprod 1996; 11(7):1558–1564.
17. Bonduelle M, Wennerholm UB, Loft A, et al. A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. Hum Reprod 2005; 20(2):413–419.
18. Ponjaert-Kristoffersen I, Bonduelle M, Barnes J, Nekkebroeck J, et al. International collaborative study of intracytoplasmic sperm injection-conceived, in vitro fertilization-conceived, and naturally conceived 5-year-old child outcomes: cognitive and motor assessments. Pediatrics 2005.
19. Bonduelle M, Wilikens A, Buysse A, et al. Prospective follow-up study of 877 children born after intracytoplasmic sperm injection (ICSI), with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. Hum Reprod 1996; 11(suppl 4):131–155.
20. Bonduelle M, Wilikens A, Buysse A, et al. A follow-up study of children born after intracytoplasmic sperm injection (ICSI) with epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. Hum Reprod 1998; 13(suppl 1):196–207.
21. Bonduelle M, Camus M, De Vos A, et al. Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. Hum Reprod 1999; 14(suppl 1):243–264.
22. Sutcliffe AG, Taylor B, Saunders K, Thornton S, Lieberman BA, Grudzinskas JG. Outcome in the second year of life after in vitro fertilisation by intracytoplasmic sperm injection: a UK case-control study. Lancet 2001; 357(9274): 2080–2084.
23. Leslie GI, Gibson FL, McMahon C, Tennant C, Saunders DM. Infants conceived using in vitro fertilization do not over-utilize health care resources after the neonatal period. Hum Reprod 1998; 13(8):2055–2059.

24. Saunders K, Spensley J, Munro J, Halasz G. Growth and physical outcome of children conceived by in vitro fertilization. Pediatrics 1996; 97(5):688–692.
25. McKibbin M, Dabbs TR. Assisted conception and retinopathy of prematurity. Eye 1996; 10(pt 4):476–478.
26. Watts P, Adams GG. In vitro fertilisation and stage 3 retinopathy of prematurity. Eye 2000; 14(pt 3A):330–333.
27. Anteby I, Cohen E, Anteby E, BenEzra D. Ocular manifestations in children born after in vitro fertilization. Arch Ophthalmol 2001; 119(10):1525–1529.
28. White L, Giri N, Vowels MR, Lancaster PA. Neuroectodermal tumours in children born after assisted conception. Lancet 1990; 336(8730):1577.
29. Kobayashi N, Matsui I, Tanimura M, et al. Childhood neuroectodermal tumours and malignant lymphoma after maternal ovulation induction. Lancet 1991; 338(8772):955.
30. Bruinsma F, Venn A, Lancaster P, Speirs A, Healy D. Incidence of cancer in children born after in vitro fertilization. Hum Reprod 2000; 15(3):604–607.
31. Lerner-Geva L, Toren A, Chetrit A, et al. The risk for cancer among children of women who underwent in vitro fertilization. Cancer 2000; 88(12):2845–2847.
32. Klip H, Burger CW, de Kraker J, van Leeuwen FE. Risk of cancer in the offspring of women who underwent ovarian stimulation for IVF. Hum Reprod 2001; 16(11):2451–2458.
33. Doyle P, Bunch KJ, Beral V, Draper GJ. Cancer incidence in children conceived with assisted reproduction technology. Lancet 1998; 352(9126):452–453.
34. Bergh T, Ericson A, Hillensjo T, Nygren KG, Wennerholm UB. Deliveries and children born after in vitro fertilisation in Sweden 1982–1995: a retrospective cohort study. Lancet 1999; 354(9190):1579–1585.
35. Stromberg B, Dahlquist G, Ericson A, Finnstrom O, Koster M, Stjernqvist K. Neurological sequelae in children born after in vitro fertilisation: a population-based study. Lancet 2002; 359(9305):461–465.
36. Healy DL, Saunders K. Follow-up of children born after in vitro fertilisation. Lancet 2002; 359(9305):459–460.
37. Leviton A, Stewart JE, Allred EN, Dammann O, Kuban K. Neurological sequelae in in vitro fertilisation babies. Lancet 2002; 360(9334):718.
38. Sutcliffe A, Peters C, Bowden S, et al. ART and imprintable disorders: British Isles Survey. Hum Reprod 2005; 20 (suppl 1).
39. Maher ER. Imprinting and assisted reproductive technology. Hum Mol Genet 2005; 14(spec no 1):R133–R138.

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Reproductive Technologies

In Vitro Fertilization

A Practical Approach

about the book . . .

Supplying a comprehensive and hands-on approach to in vitro fertilization (IVF), this source presents established state-of-the-art procedures and techniques, as well as the most current research in the field. Expert contributors also discuss the history of IVF and the potential of future research.

Offering essential information for reproductive endocrinologists, practitioners in IVF laboratories, and embryologists, this source guides readers through every step of the process, from patient pre-treatment to monitoring of outcomes...familiarizes readers with key procedures in ovarian stimulation, oocyte retrieval, assessment of gametes and subsequent fertilization, and micromanipulation procedures such as ICSI and embryo biopsy... reviews embryo culture, preimplantation genetic diagnosis, and quality management for optimal IVF outcomes...and considers methods of embryo selection and cryobiology, along with practical aspects of transfer techniques and factors influencing implantation.

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