Andrology
Laboratory Manual

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FOREWORD

It gives me great pleasure to write the foreword to this book *Andrology Laboratory Manual*. Its aim is to provide detailed information for the interdisciplinary team of specialists from the clinical members: gynecologists, endocrinologists and urologists to the clinical scientists and embryologists. It encompasses both the latest laboratory techniques and clinical treatments. Our fundamental knowledge is rejuvenated with a concise overview of spermatogenesis, sperm maturation and the genetics of male infertility. Our understanding of the complexity and vulnerability of these processes is enhanced by up-to-date information of the latest advances in male infertility evaluation. Chapters for clinicians include novel information on endocrine disorders, compromising lifestyle factors, sexual dysfunction and surgical sperm retrieval techniques. Chapters for scientists focus on laboratory skills in sperm preparation, protection from infection and cryopreservation. Its format makes it useful as an introductory text and also as an everyday reference resource for those working in the field of Clinical Andrology. I recommend it as a useful, accessible book for every fertility center.

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PREFACE

Today one can find a good number of books written on infertility, but very few which are devoted solely to male infertility and its investigations and management. Most of the books on andrology concentrate on the scientific and some specific aspects of clinical andrology without much attention to male infertility.

Application of Assisted Reproductive Technologies (ART) in dealing with male infertility is in the hands of gynecologists, physicians, urologists and embryologists. This book is an excellent reference for beginners and also for those who are practicing reproductive medicine to update their knowledge in the field of male infertility investigation and management. More importantly, the book elaborates the laboratory aspects in detail, providing reliable information to clinicians to decide the course of therapy to deal with male infertility effectively.

The *Andrology Laboratory Manual* covers a wide variety of topics including basic sperm biology, male reproductive endocrinology, physiology of sperm maturation and fertilization, genetics issues of male infertility, cryopreservation, surgical sperm retrieval, sexual dysfunction and the effect of environmental factors on semen parameters. Contributions from renowned national and international experts in the field have ensured a standard of excellence comparable to the best in the field.

This volume will serve as a textbook for newcomers to andrology while also providing the experienced physician with a valuable reference manual. It will appeal to all concerned with male reproductive health—andrologists, urologists, embryologists, endocrinologists, gynecologists, as well as basic scientists. The end aim of the book is to help in optimizing results and generate ART treatment of high quality and we hope that it works towards this end.

Kamini A Rao
Ashok Agarwal
MS Srinivas

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The editors would like to thank all those involved with the writing and publication of this book. The authors of various chapters who have taken time out from their busy schedules to contribute generously, we thank them for their excellent and well-researched articles. A special thanks goes out to Dr Alex for his excellent contributions as well as his unstinting efforts and involvement in every stage of bringing out this book.

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SECTION 1

Male Reproductive System—Anatomy and Physiology

CHAPTER 1

Anatomy and Physiology of Male Gametogenesis

Alex Varghese, Fnu Deepinder, Angali Chandra, Ang Wen Jeat, Furquan Pathan, Ashok Agarwal

ABSTRACT

Basic understanding of the male reproductive system is fundamental in effective evaluation and treatment of male infertility. This chapter is a concise introduction to the male reproductive anatomy and the intricately designed process of spermatogenesis along with its hormonal control.

INTRODUCTION

Understanding the fundamentals of anatomy and physiology of male reproductive system is a key to effective evaluation and treatment of male infertility. It comprises of the hypothalamic-pituitary-testis axis, epididymis, vas deferens, seminal vesicles, prostate and urethra.

ANATOMY OF MALE REPRODUCTIVE SYSTEM

Development

The male urinary and reproductive systems share a common developmental origin. The testes and extra-testicular ducts arise from three different tissues: *intermediate mesoderm*, *mesodermal epithelium* and *primordial germ cells*.

- The intermediate mesoderm forms a urogenital ridge that gives rise to testicular *stroma* and the *mesonephric (Wolffian) duct*.
- The mesodermal (coelomic) epithelium gives rise to Sertoli cells and the paramesonephric duct.
- The primordial germ cells migrate from yolk sac and give rise to the spermatagonia.

Sexual differentiation occurs in the seventh week of gestation in embryos carrying the Y-chromosome.

Transcription of the SRY gene present on the Y-chromosome leads to synthesis of testis-determining factor (TDF) protein. Secretion of TDF protein stimulates the nascent *Leydig cells* to produce testosterone, causing development of the mesonephric duct. It also stimulates *Sertoli cells* to secrete Mullerian-inhibiting factor (MIF), which leads to the regression of the paramesonephric duct. This cascade of events leads to the formation of male internal genital organs. Conversion of testosterone to dihydrotesto-sterone (DHT) induces the urogenital sinus to form the male external genitalia, prostate and urethra.

First step in testicular development is the formation of tunica albuginea. This layer of fibrous connective tissue separates the seminiferous cords (also known as sex cords) from the surface epithelium. These seminiferous cords are separated from one another by mesenchyme, which eventually produces the Leydig cells.

Leydig cells produce testosterone, which bind to receptors in the mesonephric duct. It helps maintain the presence of the mesonephric ducts as opposed to the female embryo where these ducts degenerate. Meanwhile, the renal corpuscle degenerates, allowing the tubules of the mesonephros to connect with the rete testis. This results in the formation of ductuli efferentes. These tubules are continuous with the mesonephric duct, and form what is known as the epididymis.

The seminiferous cords also play an important role in male differentiation. The cords contain no lumen and remain solid until puberty. They are comprised of many highly proliferative Sertoli cells that secretes

anti-mullerian hormone. This hormone inhibits the paramesonephric ducts resulting in their degeneration at approximately ninth week of development. A lumen develops in the seminiferous cords at puberty and they become seminiferous tubules.[1]

Structure-testis, Epididymis and Vas Deferens

The testes are the male gonads, similar to ovaries in females. The human testes are two glandular, ovoid organs that lie in the scrotum enveloped by a strong connective tissue covering, the tunica albuginea. Early in the embryonal life, testes lie retroperitoneally in the abdominal cavity. Before birth, the testes and spermatic cord descend through the inguinal canal into the scrotum.[1]

Each testis contains about 370 seminiferous lobules measuring about 180 µm in diameter each. These lobules lie between the fibrous septa extending between the mediastinum testis and the tunica albuginea. They are enclosed by connective tissue containing Leydig cells, blood vessels, lymphatics and nerves.

Along the posterior border, the testes are loosely connected to the epididymis, a narrow, tightly-coiled tube that connects the efferent ducts from the back of each testis to its vas deferens. Spermatozoa produced in the testis are stored in the epididymis to be carried away by the vas deferens. Smooth muscles in the wall of the epididymis contract to thrust the spermatozoa forward into the prostatic urethra. Here sperms mix with secretions from accessory glands including the prostate, seminal vesicles and bulbourethral gland[2,3] (Figures 1.1 and 1.2).

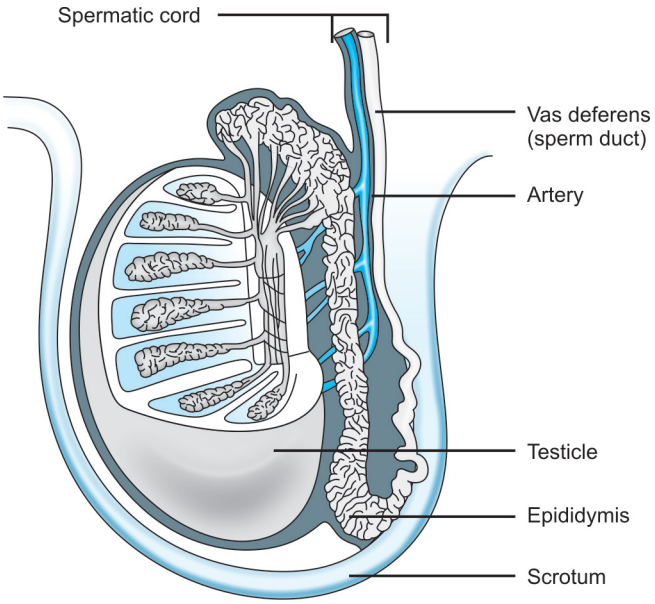


Figure 1.1: Diagram of male reproductive system

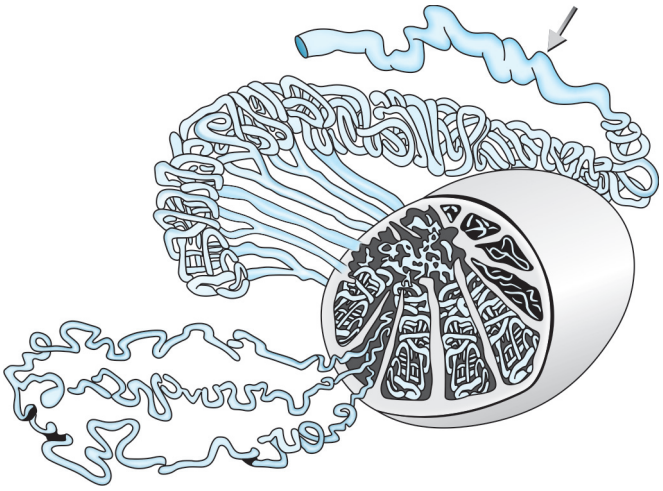


Figure 1.2: Cross-sectional view of testis

Supporting Cells

- Leydig cells
- Sertoli cells

Leydig Cells

Leydig cells, named after the German anatomist Franz Leydig who first identified them in 1850, are somatic cells lying in the testicular interstitium. These are irregularly shaped cells containing granular cytoplasm and are often seen in clumps within the connective tissue. Leydig cells of testis are the main site of synthesis and secretion of androgens, including testosterone, the primary male sex hormone.[1] Luteinizing hormone (LH) secreted by the pituitary, stimulates the Leydig cell to produce testosterone, which is then accumulated in the interstitium and seminiferous tubules.

Sertoli Cells

The Sertoli cells line the seminiferous tubules and are the 'nurse' cells of the testes. Their main function is to nurture the developing germ cells during various stages of spermatogenesis.[4] Sertoli cells communicate with germ cells through multiple sites for the maintenance of

spermatogenesis. Sertoli cells also form tight junctions that divide the seminiferous tubules into two compartments for the spermatozoal development. The basal compartment below the tight junctions is in contact with the circulatory system and is the site where spermatogonia develop into primary spermatocytes. The tight junctions open at specific times and allow progression of spermatocytes to the adluminal compartment, where meiosis is completed. In the adluminal compartment, spermatocytes are protected by a blood-testis barrier formed by tight junctions between the Sertoli cells.

The principal functions of Sertoli cells are as follows:

- Provide support for germ cells, forming an environment in which they develop and mature.
- Provide the signals that initiate spermatogenesis and sustain spermatid development.
- Regulate pituitary gland function and, in turn, control of spermatogenesis.
- Secrete aqueous secretion into the lumen to aid sperm transport.

SUMMARY

Testis

- 90% seminiferous tubules (Sertoli and germ cells).
- 10% interstitial (Leydig cells, connective tissue, blood supply).
- Two functions:
  - Spermatogenesis—in the seminiferous tubules.
  - Biosynthesis of testosterone (T)—Leydig cells.

Accessory Glands[3]

- Seminal vesicle—60% of semen.
  Contain fructose which is the energy source for sperm; and prostaglandins that react with cervical mucus and induce peristaltic contractions up the tract.
- Prostate—20% of semen.
  Alkaline solution; contain citrate, cholesterol, and prostaglandins.
- Bulbourethral glands
  Buffers to neutralize the acidic environment of the reproductive tracts; contain phosphate and bicarbonate.

Biosynthesis of Testosterone (T)—Leydig Cells

- Synthesized from cholesterol.

Spermatogenesis

Spermatogenesis is the process by which male spermatogonia develop into mature spermatozoa.

During this complex process, primitive totipotent stem cells divide to produce daughter cells, which, over a span of approximately 70 days mature into spermatids. The process involves both mitosis and meiosis and is regulated by Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) from the anterior pituitary.

Spermatogonia divide by mitosis and differentiate until they become primary spermatocytes, which remain dormant until puberty.

The process of spermatogenesis can be best understood by discussing individual stages.

The first stage of formation of spermatozoa is *spermatocytogenesis.* During this stage, stem cells divide to produce a population of cells destined to become mature sperm cells and to replace themselves. Spermatocytogenesis occurs in the basal compartment.

Spermatogonium is of three functional types (Figure 1.3)

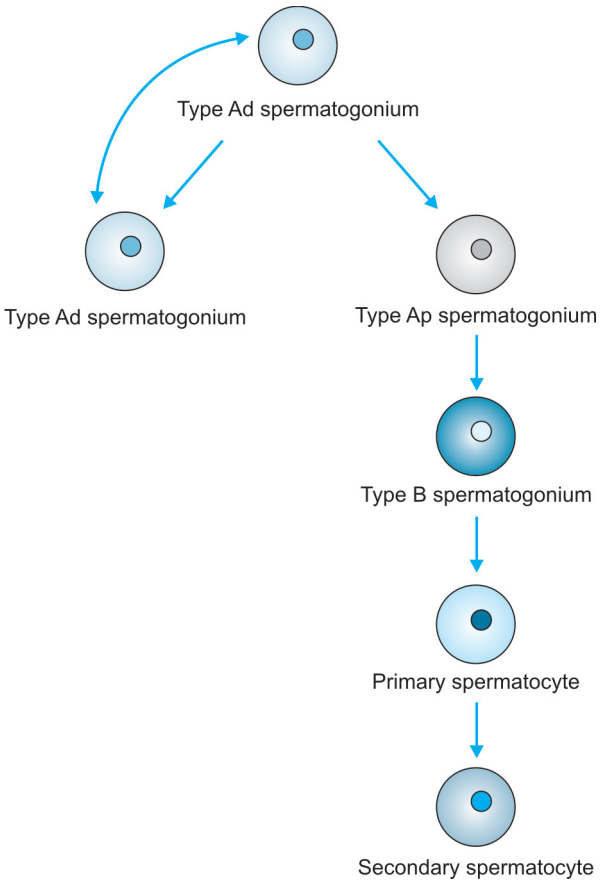


Figure 1.3: Spermatocytogenesis

- Type Ad "dark"
- Type Ap "pale"
- Type B

Type Ad cells maintain the initial pool of spermatogonium. These cells do not take part directly in the process of spermatid formation but ensure a continuous supply of stem cells for spermatogenesis. Type Ad cells are capable of dividing into—Type Ap and Type Ad cells itself.

Type Ap spermatogonia undergo repeated mitotic divisions to produce a clone of cells. These cells are tethered together with cytoplasmic bridges that allow synchronized development. The resultant cells differentiate into Type B spermatogonia.

Type B spermatagonia undergo mitosis to produce diploid intermediate cells, the primary spermatocytes. The primary spermatocytes are arrested in the prophase of the first meiotic division until puberty and thus have the longest life span of all types of spermatagonia. At puberty, the diploid (2N) primary spermatocytes enter meiosis I and divide by to become haploid (N) secondary spermatocytes.

Secondary spermatocytes have the shortest life span (1.1 to 1.7 days) of all types of spermatagonia.

Secondary spermatocytes undergo meiosis II to yield spermatozoa (N) with half the DNA material of the primary spermatocytes from which they originated. The process begins with one primary spermatocyte containing double genetic material, which divides into two haploid secondary spermatocytes, each containing normal complement of genetic material and finally resulting in four spermatids, each containing half of the genetic material from the original spermatocyte (Figure 1.4).

Once the process of meiosis is completed, the final stage of *spermiogenesis*, begins. During this stage the spermatids develop into mature, motile spermatozoa (Figure 1.5). This occurs in deep folds of cytoplasm of the Sertoli cells. The maturation of spermatids to spermatozoa depends on the action of androgens on the Sertoli cells in which the developing spermatozoa are embedded. FSH acts on the Sertoli cells to facilitate the last stages of spermatid maturation.

Six different stages in the process of spermatid maturation are described by morphology:

- Sa-1 and Sa-2
  - Golgi complex and mitochondria are well developed and differentiated.
  - The acrosomal vesicle appears.

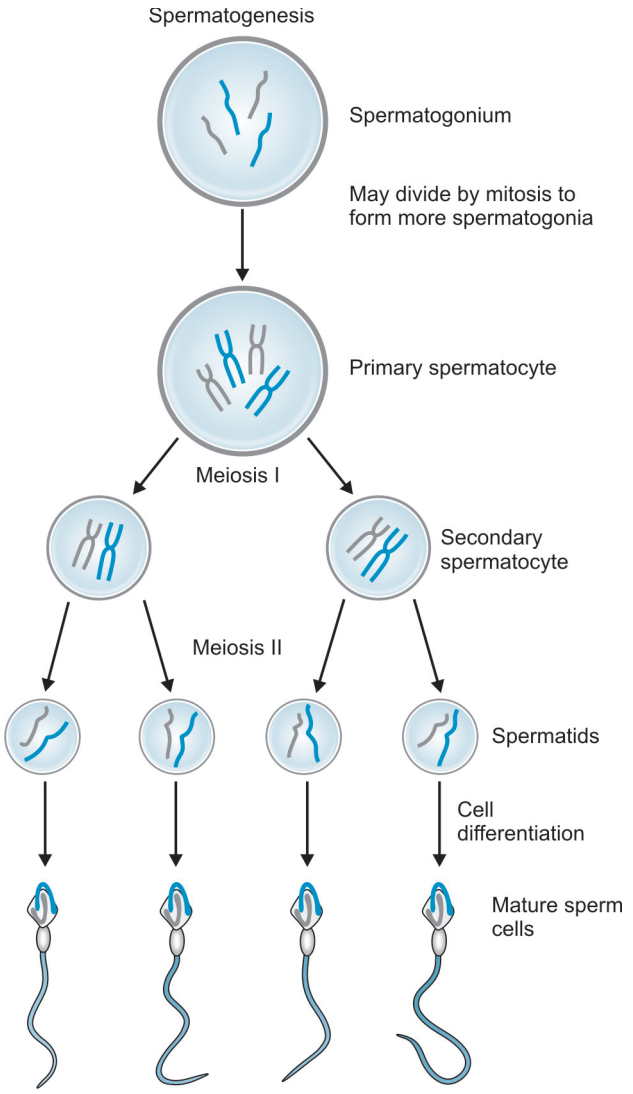


Figure 1.4: Spermatogenesis

  - The chromatin body appears in one pole of the cell opposite from the acrosomal vesicle.
  - The proximal centriole and axial filaments appear.
- Sb-1 and Sb-2
  - Acrosome formation is completed.
  - Intermediate piece is formed.
- Sc-1 and Sc-2
  - Tail development is completed during Sc stage.

During the post meiotic phase, progressive condensation of the nucleus occurs with inactivation of the genome, the histones convert to transitional proteins, and protamines convert to well-developed disulfide bond.[5]

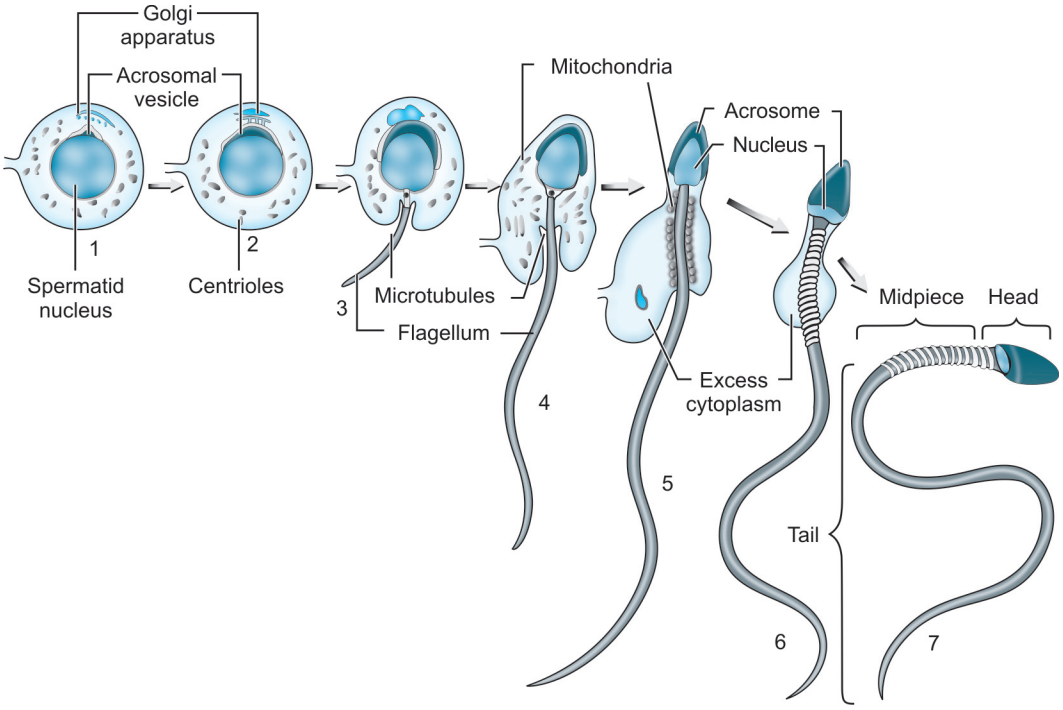


Figure 1.5: Spermiogenesis

Mature spermatozoa are released from the Sertoli cells and become free in the lumen of the tubules. This process is *spermiation*.

The newly released sperm are non-motile. They are suspended in a fluid secreted by the Sertoli cells and are transported to the epididymis by peristaltic contraction of the myoid cells present in the walls of the tubules. The spermatozoa reach the epididymis by passing through the efferent ductules, the first segment of the extra-testicular duct system. Within the epididymis, activation of the CatSper protein localized in the principal piece of the sperm tail develops the progressive motility of the sperm. This protein appears to be a Ca^{2+} ion channel that permits cAMP-generated Ca^{2+} influx. Smooth muscle peristalsis transports the sperm through the remainder of the male reproductive system.

Spermatozoa

Each sperm is an intricate motile cell, rich in DNA, with a head comprised mostly of chromosomal material (Figure 1.6). Sperm are highly specialized, differentiated and condensed cells that do not divide. Approximately 60 µm long and 1 µm wide, each sperm is composed of the head, midpiece (body), and tail.

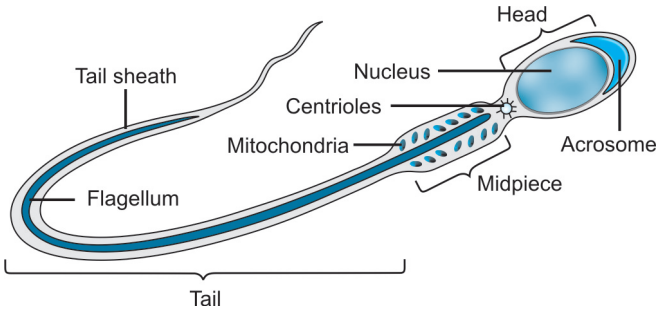


Figure 1.6: Schematic diagram of spermatozoa

Head

The normal head of the spermatozoa is oval and measures about 3.0-5.0 µm in length and 2.0-3.0 µm in width with a thickness of 1.5 µm (Mortimer). The normal length-to-width ratio is about 1.50-1.75 (Anibal AA). The head contains the nuclear material for the fertilization process. The acrosome covers the sperm head like a cap. This lysosome-like organelle is rich in enzymes which mediate the penetration of ovum by the sperm.

Neck

The neck is the junction between the head and tail. The presence of decapitated spermatozoa is a common abnormality.

Tail

The tail contains the locomotory flagellum, divided into middle, principal and end pieces. The middle piece has the flagellum, surrounded by a sheath of mitochondria that provide the energy for movement.

Thermoregulation of the Scrotum

The male gonads lie in the scrotum which is a characteristic feature of almost all mammals. Human testicular temperature is physiologically maintained within a range of 32-35°C. The location of testes in the scrotum facilitates the production of viable and mature spermatozoa in a comparatively cooler environment than the rest of the body.[6]

Impairment of spermatogenesis by elevation of testicular temperature is now a widely accepted phenomenon.[7]

Mieusset et al reported that an increase of 1.5-2°C in scrotal temperature inhibited spermatogenesis.

In man, dangling scrotum helps thermoregulation by allowing the heat produced during spermatogenesis to dissipate.[6] Other supporting features of the scrotum such as thin skin with high vascularization, scanty hair, abundant sweat glands and absence of subcutaneous fat also facilitate the heat dissipation.

Excessive heat in the testes or exposure to warm, ambient temperature cause the scrotal skin to loosen, thus increasing its surface area; and the scrotal muscles to relax so as to take the testes away from body thereby preventing sperm damage.[8] On exposure to cold, the scrotal surface area is minimized by rugosities, and cremaster muscles lift the testes closer to the abdomen in order to conserve heat and protect the sperm. The absence of spermatogenesis in cryptorchidism where the testes is retained intraabdominally has been attributed to supraphysiological temperatures.

Varicocele, a tortuous dilation of testicular veins resulting in stagnation of venous blood in the pampiniform plexus, is the leading causes of secondary male infertility. Hyperthermia has been identified as one of the major factors contributing to sperm damage in varicocele. Although controversy exists behind this hypothesis, but the scientific evidence is strong.

Functions of the Epididymis in Spermatogenesis

Sperm maturation is defined as the development of the ability of spermatozoa to fertilize eggs as they progress through the epididymis. The epididymis has been shown to play an important role in sperm maturation, serving as the motility gaining site.

Several small water-soluble components of epididymal fluid (myo-inositol, L-carnitine, taurine, glutamate) are taken up by spermatozoa during post-testicular maturation, to function as a reserve of intracellular osmolytes against the osmotic challenges that spermatozoa experience later at ejaculation. As a result, the composition of the fluid environment within the epididymis differs significantly from that of plasma. Inorganic ions, rather than organic compounds, seem to be the major osmolytes. The effects of high osmolality in dehydrating spermatozoa as a means of enforcing sperm quiescence have been proposed, although this implies that the solutes remain extracellular. Several proteins also are found within the lumen, some of which have are known to play a role in sperm maturation. For many others, their role is still undefined.

Transport through the epididymis takes approximately a week. The transport is achieved by contraction of smooth muscles around the epididymal epithelium, aided by continuous fluid movement. The sperm attain different functions at different parts of the epididymis. Motility is attained as spermatozoa pass through the caput (head) region, and fertilizing ability is achieved as the spermatozoa pass through the corpus (body).[3] Considerable absorption of water occurs in the proximal region. The cauda region stores the spermatozoa until ejaculation. This is the site of aspiration of sperm for ICSI (Figure 1.7).

Tight junction complexes between the epididymal cells form the blood-epididymis barrier, an important

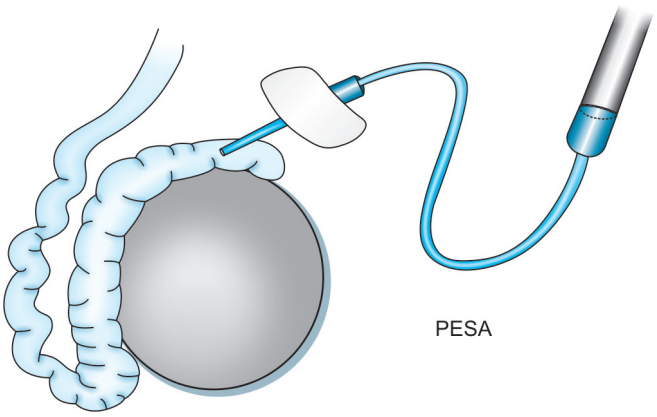


Figure 1.7: Percutaneous epididymal sperm aspiration

physiological and anatomical barrier that also gives immunological protection to the spermatozoa. Spermatozoa are immunogenic and must be protected from the immune system.

The epididymis also possesses the ability to protect spermatozoa from oxidative attack while stored in the cauda region, through the local actions of antioxidants such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD). The eventual result of activity within the epididymis is the production of fully viable spermatozoa capable of fertilizing the ovum.[9]

HORMONAL REGULATION OF SPERMATOGENESIS

Spermatogenesis is regulated by hormones secreted by the hypothalamic-pituitary-gonadal axis. Spermatogenesis is regulated by negative feedback mechanism.

Hypothalamus secretes gonadotrophin releasing factor (GnRH) in the hypothalamo-hypophyseal portal circulation. This factor stimulates synthesis and releases the gonadotrophins, FSH and LH, by the pituitary gland into the systemic circulation.

LH acts on the Leydig cells stimulating the production of testosterone. FSH acts on the Sertoli cells and is important for the development of the Sertoli cells that are vital for spermatogenesis. Sertoli cells under the influence of the FSH secrete androgen binding protein, inhibin and plasminogen activator. Androgen binding protein (ABG) is necessary to maintain high levels of the androgens locally which is important for spermatogenesis. The plasminogen factor helps in spermiation and inhibin has a negative feedback effect on the FSH secretion by the anterior pituitary gland.[10]

Testosterone is the principle androgen produced by the Leydig cells in the testis under the influence of LH.

Functions of Testosterone

- Differentiation, development and maturation of internal and external reproductive organs in male.
- Stimulation of spermatogenesis.
- Regulation of accessory sex gland functions.
- Development of the secondary sex characters.
- Regulation of gonadotrophin secretion by negative feedback mechanism.

REFERENCES

1. Akhmerova LG. Leydig cell development. Usp Fiziol Nauk. 2006;37(1):28-36.
2. de Kretser DM, Huidobro C, Southwick GJ, et al. The role of the epididymis in human infertility. J Reprod Fertil Suppl. 1998;53:271-5.
3. Elzanaty S, Richthoff J, Malm J, et al. The impact of epididymal and accessory sex gland function on sperm motility. Hum Reprod. 2002;17(11):2904-11.
4. Johnson L, Barnard JJ, Rodriguez L, Smith EC, Swerdloff RS, Wang XH, et al. Ethnic differences in testicular structure and spermatogenic potential may predispose testes of Asian men to a heightened sensitivity to steroidal contraceptives. J Androl. 1998 May 1;19(3):348-57.
5. Karpenko NO, Bondarenko VA, Kavok NS, et al. The maturation of the spermatozoa: events, consequences and possible ways of control. Fiziol Zh 2007;53(1):91-103.
6. Mieusset, R and Bujan L. Testicular heating and its possible contributions to male infertility: A review. Int J Androl. 1995;18(4): 169-84.
7. Schuppe HC, JA. Influence of genital heat stress on semen quality in humans. Andrologia 2007;39(6):203-15.
8. Skandhan KP, Rajahariprasad A. The process of spermatogenesis liberates significant heat and the scrotum has a role in body thermoregulation. Med Hypotheses 2007;68(2):303-7.
9. Potts RJ, Jefferies TM, Notarianni LJ. Antioxidant capacity of the epididymis. Hum Reprod. 1999;14(10):2513-6.
10. Wu X, Wan S, Lee MM. Key factors in the regulation of fetal and postnatal leydig cell development. Journal of Cellular Physiology 2007;213(2):429-33.

CHAPTER 2

Human Spermatozoa—Production, Migration and Fertilization

Sathish Adiga

Testes and Sperm Production

Human testes are seated in the scrotum and weighs approximately about 0.08% (10.5 to 14 g) of the body weight. Testis has both endocrine and gametogenic functions which are controlled by gonadotrophic hormones secreted from pituitary. The glandular part of the testis consists of 200-300 lobules containing seminiferous tubules and the loose connective tissue containing contractile peritubular myoid cells and clusters of steroid producing Leydig cells.

The Leydig cells are large polyhedral cells with an accentric nucleus containing one to three nucleoli. Their cytoplasm contains a considerable amount of smooth endoplasmic reticulum, lipid droplets and unique needle shaped crystalloid inclusions (crystals of Reinke). They synthesize and secrete the male sex steroid hormone testosterone under the influence of pituitary LH. The secreted hormone upon release in to the bloodstream regulates its own production by negative feedback inhibition on the pituitary to modulate the LH secretion.

Sertoli cells are variable in shape and extend from basement membrane of the tubule wall to tubular lumen. Sertoli cells are in continuous contact with spermatogenic cells at various developmental stages until they are mature enough for release. Sertoli cells are sensitive to FSH which secretes androgen binding protein (ABP), which has a high affinity for testosterone and plays an important role in the transport and localization of the androgens within the tubule. In addition, sertoli cells secrete a polypeptide known as inhibin which provides a negative feedback mechanism on the pituitary to regulate FSH secretion.

Spermatogenesis

Spermatozoa are produced in the testes by a process known as spermatogenesis. The first spermatozoa are released at puberty, but these represent the culmination of events that begin early in fetal life. In the seminiferous tubule there are two types of somatic cells—the myoid or smooth muscle like cells and sertoli cells, and different types of germ cells- spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatids, elongating and elongated spermatids. On the basis of cell and nuclear dimensions, distribution of nuclear chromatin and histochemistry, three types of spermatogonia are identified in human testis. Dark A type (Ad), pale type A (Ap) and type B. Ad cells divide mitotically to maintain the population of spermatogonia. Some divisions give rise to Ap cells which also divides mitotically but remain together in clusters by fine cytoplasmic bridges. These are the precursors of type B cells which are committed to the spermatogenic sequence. The type B cells enter the final round of DNA synthesis, without undergoing cytokinesis, finally leave the basal compartment and cross the blood-testis barrier to enter the meiotic prophase as primary spermatocytes.

Primary spermatocytes have a diploid chromosome number but duplicated sister chromatids. Primary spermatocytes are characteristically large cells with large round nuclei in which the nuclear chromatin is condensed into dark thread like coiled chromatids at different stages in the process of crossing over and genetic exchange between chromatids of maternal and paternal homologs. These cells give rise to secondary spermatocytes with the haploid chromosome complement. Few secondary

spermatocytes are seen in tissue sections because they rapidly undergo the second meiotic division, where sister chromatids separate to form haploid spermatids. Theoretically each primary spermatocyte produces four spermatids, but some may degenerate during maturation.

Spermiogenesis

Spermatids do not divide again but gradually mature into spermatozoa by a series of nuclear and cytoplasmic changes known as spermiogenesis. All these maturational changes take place while the spermatid remains closely associated with sertoli cells and linked by cytoplasmic bridges with each other. The nuclear histones are replaced by protamines and the chromatin becomes highly condensed. Disulfide bond cross-linking of the protamines makes the inert, condensed chromatin resistant to DNAse. The first phase of spermiogenesis is the Golgi phase during which spermatids begin to develop polarity and hydrolytic enzymes accumulate in Golgi vesicles. At the other end, it develops a thickened mid-piece where mitochondria gather and form an axoneme. In the cap phase, the acrosomal vesicle flattens and envelopes the anterior half of the nucleus to form an acrosomal cap. During the acrosome phase, one of the centriole elongates to become the sperm tail. In addition, the developing spermatozoa orient themselves so that their tails point towards the center of the lumen, away from the epithelium. In the final phase of maturation excess cytoplasm is detached as a residual body which is phagocytosed and degraded by Sertoli cells. Residual cytoplasm is shed from the neck region of the mature spermatid as it is released from the seminiferous epithelium into the tubule lumen, a process termed spermiation. A small residual cytoplasmic droplet also remains attached to testicular spermatozoa at the point of separation. As the cell undergoes further maturation during epididymal transit this cytoplasmic droplet migrates along the tail and is finally lost.

Human sperm production takes a fixed period of 70 ± 4 days from the commitment of type a spermatogonium to undergo spermatogenesis to the appearance of mature spermatid in the seminiferous epithelium. This rate cannot be altered by exogenous hormonal treatment, extrinsic factors such as temperature, or noxious agents such as radiation. Release of the testicular spermatozoan and its passage through the excurrent ducts of the testis and the epididymis takes around 12 to 21 days. Consequently a period of 10 to 12 weeks is required for the production of human spermatozoa from germinal stem cells to a stage where they are ready to be ejaculated.

Epididymis

Epididymis is divided into three functionally distinct regions- caput (head), corpus (body) and cauda (tail) epididymis. Epithelial cells of epididymis are androgen sensitive and have both absorptive and secretory functions. Most of the testicular fluid is resorbed by the caput which helps in concentration of spermatozoa (10-100 fold). Epididymal epithelium secretes epididymal plasma in which the spermatozoa are suspended until ejaculation. The composition of epididymal fluid changes along the length of epididymis. The epididymis has following major functions.

1. *Sperm transport:* An obvious function of the epididymal tubule is to transport sperm from the testis to the vas deferens. Net fluid transport rates are rapid in the proximal epididymis, where fluid is non-viscous and water is being rapidly absorbed from the lumen, but transport rates slow considerably in the distal tubule,[1] where the lumen content can be quite viscous and little water absorption is continuing. The time required for this transport has been assessed in a variety of ways,[2] and is relatively short in humans (2-6 days), a feature that may relate to the relative ease with which some human sperm are matured. The propelling forces in the epididymis are: 1) hydrostatic pressure from fluid secretion in the testis and 2) peristaltic contractions of the tubule.[2] The total sperm transport time from the seminiferous tubule to the exterior in sexually active men is about 10-14 days.
2. *Sperm concentration:* The sperm concentrating effect of epididymis is due to fluid reabsorption subsequent to antiluminal electrolyte transport.[3] Catecholamines,[4] aldosterone,[5,6] and the renin-angiotensin system[7] play a regulatory role in this fluid reabsorption.
3. *Sperm storage:* Approximately 70% of all spermatozoa present in male reproductive tract are stored in caudae epididymis. However, it is not a perfect organ for sperm and hence the viability of spermatozoa decreases with increase in abstinence period. Spermatozoa first lose their fertilizing ability followed by motility and finally the vitality. Therefore for accurate semen analysis a minimum sexual abstinence of three days is advisable.

Sperm Maturation

As the spermatozoa leave the testicular environment, they undergo dramatic changes in their morphology, metabolic activity and biophysical activity. These changes are obligatory for its behavior in female reproductive tract and to attain the fertilizing ability. Spermatozoa taken from caput or proximal corpus are generally incapable of fertilization, whereas those from the distal corpus and the cauda are able to undergo capacitation and achieve normal gamete interaction.[8]

The most striking change observed in spermatozoa during epididymal transit is acquisition of motility. Metabolically they become very active and exhibit increased fructolytic activity. Spermatozoa recovered from the caput epididymis show immotility or slight vibratory activity of the tail. As it progresses further in the epididymis, the motility increases. Spermatozoa stored in the cauda are capable of normal progressive motility upon their entry into seminal plasma.

During the transit through epididymis there are no obvious changes in the morphology of the tail. But the cytoplasmic droplet observed around the neck of spermatozoa during spermiation slowly migrates caudally down the mid piece which is completely lost during the time of ejaculation. The droplet contains the membrane bound vesicles and other elements, the function of which are not clearly understood. Cytoplasmic droplet is involved in estrogen production[9] and the ability to develop the regulatory response to adjust to osmotic changes during maturation.[10] However, the presence of cytoplasmic droplet in the ejaculated spermatozoa can lead to DNA damage through oxidative stress.[11] Changes also occur in sperm head during epididymal transit. The acrosome attains its final shape and the reorganization of the acrosomal contents takes place which is necessary for gamete interaction and fertilization. The sperm chromatin condenses further by increased stabilization due to the formation of disulfide bonds. Sperm plasma membrane changes are recorded based on the light-reflecting properties, uptake of the vital dyes, binding of lectins, adhesiveness, net charge and density and agglutination pattern.

Accessory Sexual Glands

Seminal vesicles: The paired seminal vesicles lie on the dorsal face of the bladder. Each is a convoluted glandular sac or tube about 5 -6 cm in length arising from the vas deferens just below the ampulla of the vas. The yellowish, viscous, alkaline secretion of the seminal vesicles is called the vesicular fluid. It is the last fraction of the semen ejaculated and contributes about 70% of the ejaculate. The major constituents of seminal fluid are fructose, ascorbic acid, prostaglandins, inorganic phosphorous and potassium. Congenital absence of vas deferens is usually associated with agenesis of seminal vesicles due to their common embryonic origin.

Prostate: It is the largest accessory gland and completely surrounds and completely surrounds the urethra below the neck of the bladder. It secretion is acidic (pH 6.8), homogeneous, serous and slightly milky which contributes to approximately 30% of the ejaculate. The prostatic fluid is rich in fibrinolytic enzymes and prostate specific antigen responsible for liquefaction of coagulated vesicular secretion. All the citric acid, acid phosphatase, sodium and zinc found in the ejaculate come from prostate. Prostatic fluid also enhances the motility of epididymal and ejaculated spermatozoa by contributing certain factors like albumin.

Bulbourethral (Cowpers) glands: The two small lobulated glands are located dorsal to the bulb of the penis whose excretory ducts open into the penile urethra. The secretions of these glands are clear and rich in sialoprotein. It is secreted during erection and just prior to ejaculation of spermatozoa. The primary function of this secretion is to lubricate the urethra, which is probably better expressed as the neutralization of any acidic urinary residue in the urethra before ejaculation.

Ejaculation and Insemination

Until ejaculation the spermatozoa are stored in the caude epididymides. During ejaculation the spermatozoa stored (contributes to 5-15% of ejaculate volume) are mixed with the secretions of the accessory glands and then emitted to the exterior along the penile urethra with the aid of bulbourethral secretions. The first part of the ejaculate rich in prostatic secretion is acidic, less viscous and contains mostly viable spermatozoa. The second fraction is transitional between prostatic and vesicular fluid, which is alkaline, viscous and may have poor quality spermatozoa. The last fraction usually contains only the residual spermatozoa mobilized from the epididymal reserves suspended in vesicular fluid.

Spermatozoan

Spermatozoa are highly condensed, specialized cells which do not divide. Unlike most of the cells, it has a

large, highly condensed nucleus with negligible amount of cytoplasm. Men are unique among mammals in the degree of morphological heterogeneity of the ejaculate. Normal fertile men produces as many as 50% sperm with abnormal morphology displaying defect in one or more region of sperm including size and shape of the head, chromatin condensation, presence of nuclear vacuoles, persistent cytoplasmic droplets and tail defects.

Sperm Morphology

The length of human spermatozoa is around 60 µm, out of which only 4-5 µm correspond to the head, and the remaining 55 µm correspond to the neck region and tail. The normal head is oval in shape with a length of 4-5 µm, width of 2.5-3.5 µm and a height of 1.5 µm. The neck region is small (around 1 µm) which connects the head and middle piece of the tail. The tail can be divided into three regions- middle piece, principal and terminal piece. The width of tail decreases progressively. The middle region is 4 µm in length and 0.4 µm in width. It shows from 8-12 helicoidal rings that correspond to the internal distribution of the mitochondria. A constriction called annulus is formed between the middle and principal piece. The principal region is around 45 µm long and characterized by its smooth surface. The narrow terminal region of the tail corresponds to the last 5 µm of the tail.

In the cephalic region of the sperm head, human spermatozoa have a well defined acrosomal region comprising about two thirds of the anterior head area.[12] It does not exhibit an apical thickening like many other species, but shows a uniform thickness/thinning towards the end forming the equatorial segment. Because of this thinning, the area will appear more intensely stained when examined with light microscope. It has homogeneous and electron dense contents rich in enzymes with proteolytic and glycolytic property (acrosine and hyaluronidase) which are required for digestion of cumulus cells and the zona pellucida during gamete interaction.

The nucleus of the head is small in size, extremely dense and covers the entire space of the head. The nucleus has an ovoid shape with a space in its posterior region where structures related to flagellum are present. Spermatozoa are haploid and carries 22 autosomes with either X or Y chromosome. The Y-bearing spermatozoa contain 4.3% less DNA than X-bearing spermatozoa.[13] The nuclear membrane shows pores only in the posterior region, but not in the acrosomal or post-acrosomal region. Close to the flagellum, the membrane is crossed by dense particles separated by a distance of 6 µm that correspond to the basal plate.

The neck or the connecting piece contains proximal centriole and the accessory structures- striated columns and capitulum. Mature sperm has a single proximal centriole found in the middle of the neck perpendicular to the longitudinal axis of the spermatozoa. Extending back from the capitulum are nine distinctively segmented columns, which at their further ends overlap the tapering ends of nine dense outer fibers of the flagellum, to which they are firmly attached. Neck is a quite slender region and there are no organelles except one or two longitudinally oriented mitochondria.

The tail or the flagellum is usually divided into a mid piece, principal piece and end piece. The mid piece extends from the head to as far as the end of the mitochondrial helix. At the center of the tail is the axoneme or axial filament complex. It consists of two central microtubules surrounded by a cylinder of nine evenly spaced (18 nm) doublet microtubules (9 + 2 arrangement). Each doublet is linked by two slender nexin links. The arms of the doublet are made up of dyenin—a large protein with ATPase activity and responsible for the movement of flagellum.

The principal piece consists of a number of circumferentially oriented ribs running between two longitudinal columns, which extend along the entire length at either side of the tail. As the sperm tail tapers, the columns become smaller and the ribs thinner, the fibrous sheath ends several microns from the tip. The last 5 nm of the tail correspond to the terminal piece. In this region, the 9 + 2 structure has been lost, and the tail contains only some microtubules. Disorganization of axonema is a frequent tail anomaly. The most common one are loss of some doublets, loss of central microtubules (9 + 0 syndrome) which leads to absolute immotility.

Sperm Motility

Testicular spermatozoa of humans have faint or no motility which is apparently due to the immaturity of the plasmalemma. During passage through the epididymis, spermatozoa undergo substantial maturational changes that result in their acquisition of motility. After ejaculation when the spermatozoa are mixed with the secretion of the accessory glands spermatozoa undergo motility activation.

Dyenin, which is a flagellar protein located in the arms of the microtubule doublet with ATPase activity generates the mechanical force for the sperm motility. The assessment of sperm motility in the ejaculate is an important functional parameter of routine semen analysis. Different grades of motility can be scored by light microscopic examination.[14]

Grade a—Rapid progressive motility (speed of ≥ 25 µm/s at 37°C)

Grade b—Slow or sluggish progressive motility

Grade c—Non-progressive motility (speed of <5 µm/s)

Grade d—Immotility

Motility assessments by simple light microscopic method is highly error prone with a large degree of inter individual and intra-individual variation. More accurate methods of motility assessment have been tried using computer aided sperm analysis (CASA). However, it is more expensive and its practical applicability in routine andrology laboratory is still under question.

Sperm Motility in Female Reproductive Tract

Passage of sperm through the female reproductive tract is regulated to maximize the chance of fertilization and ensure that sperm with normal morphology and vigorous motility will be the ones to succeed. Because sperm are terminally differentiated cells, deprived of an active transcription and translation apparatus, they must survive in the female reproductive tract. Sperm are subjected to physical stresses during ejaculation and contractions of the female tract, and also subjected to oxidative damage. They may encounter with immune system of female body since they are allogenic to female.[15] Out of millions of sperm inseminated at coitus, only a few thousand reach the Fallopian tubes and, ordinarily, only a single sperm fertilizes an oocyte.

Sperm deposition: In humans spermatozoa ejaculated at coitus are deposited most likely in the anterior vagina near the cervical os and in the posterior fornix of the vagina. Human semen coagulates within about a minute of coitus and then is enzymatically degraded in 30 min to 1h.[16] The coagulum serves to hold the sperm at the cervical os[17] and protects spermatozoa from harsh vaginal environment.[18] The coagulum is predominantly made up of semenogelin I, semenogelin II, and glycosylated form of semenogelin II, secreted by the seminal vesicles.[19] Baker and Bellis[20] examined the characteristics of sperm loss from the vagina following coitus ('flowback') occurred in 94% of copulations. The gel is degraded by prostate-specific antigen (PSA), a serine protease secreted by the prostate gland.[21] Within minutes of liquefaction, sperm begin to leave the seminal pool and swim into the cervical canal.[22] Some motile sperm may remain in the vagina for up to 24 h.

Seminal plasma has a strong buffering capacity which is able to raise the acidic pH of vagina (≤5) within seconds of ejaculation. In addition, seminal plasma contains inhibitors of immune responses, including protective components that coat the spermatozoa.[23] These are most effective when sperm are bathing in seminal plasma and may be gradually shed when sperm leave the seminal plasma behind. Cellular immunity of female reproductive tract is partially overcome by inseminating many sperm.

Spermatozoa in the Cervix

Sperm of humans enter the cervical canal rapidly where they encounter with cervical mucus. Under the influence of estrogen the cervix secretes highly hydrated mucus, often exceeding 96% water in women and the extent of hydration is correlated with sperm penetrability.[24] In luteal phase, under the influence of progesterone mucus becomes viscous and rubbery which is unfavorable for sperm motility. Other factors which influence the sperm migration through cervix include muscular activity in the vaginal and cervical walls, structure of cervical mucus and the presence of cervical crypts. Sperm may also be guided through the cervix by the microarchitecture of the cervical mucus. Mucin, the chief glycoproteins present in the mucus is responsible for viscosity of the mucus, while elasticity results from the entanglement of the molecules.[25] Human sperm have been demonstrated to orient themselves and swim through cervical mucus in a straighter path than they do in seminal plasma or medium.[26] There are three phases of sperm transport in the cervical mucus—

a. Initial rapid phase—small number of spermatozoa that reach the fallopian tube within 5 min of coitus
b. Colonization phase—a large number of spermatozoa enter the crypts of cervical glands and remain there for a prolonged period of time
c. Prolonged phase of transport—glands of the cervical crypts permit the release of sperm

Cervical mucus presents a physiologically favorable environment for prolonged sperm storage and protects

from phagocytic attack. Vigorously motile sperm have been recovered from the human cervix up to 5 days after insemination,[27] and the presence of sperm in midcycle cervical mucus forms the basis of the 'post coital test' (PCT).[28] Cervical mucus presents a greater barrier to sperm with abnormal morphology and poor motility thus acting as a means of sperm selection.[29] Compact microarchitecture of the cervical mucus at its border offers the greatest barrier to spermatozoa.[30]

Like the vagina, the cervix can mount immune responses. Vaginal insemination stimulates the migration of leukocytes, particularly neutrophils and macrophages, into the cervix as well as into the vagina.[31] Neutrophils migrate readily through midcycle human cervical mucus.[32] Immunoglobulins, IgG and IgA, have been detected in human cervical mucus. The immunoglobulins provide greater protection from microbes at the time when the cervical mucus is highly hydrated and offers the least resistance to penetration. However, when there are antibodies present that recognize antigens on the surface of ejaculated sperm, infertility can result.[15] Complement proteins are also present in cervical mucus,[33] along with regulators of complement activity.[34] Thus, there is a potential for antibody-mediated destruction of sperm in the cervical mucus as well as leukocytic capture of sperm. Some anti-sperm antibodies are not complement-activating; however, they can still interfere with movement of sperm through cervical mucus by physical obstruction.[15,35]

Spermatozoa in the Uterus

The human uterine cavity is relatively small and could be traversed in less than 10 min by sperm swimming at about 5 mm/min, which is the swimming speed of sperm in aqueous medium.[36] Transport of sperm through the uterus is likely aided by pro-ovarian contractions of the myometrium. Ultrasonography of the human uterus has revealed cranially directed waves of uterine smooth muscle contractions that increase in intensity during the late follicular phase.[37] Seminal plasma constituents especially prostaglandins are capable of causing the smooth muscle contractions in the uterus.

Rapid transport of sperm through the uterus by myometrial contractions can enhance sperm survival by propelling them past the immunological defenses of the female. When sperm first enter the uterus, they outnumber the leukocytes. As time passes, the leukocytes begin to outnumber the sperm. Also, as sperm lose protective seminal plasma coating, they may become more susceptible to leukocytic attack.[23]

Transport through the Uterotubal Junction

The number of spermatozoa ejaculated does not have a great influence on the number of sperm reaching the fallopian tube. The narrow lumen of the uterotubal junction presents anatomical, physiological barriers to sperm passage. Within the lumen of the junction, there are large and small folds in the mucosa.[38]

Transport in the Fallopian Tube

Sperm transport within the tube appears to be a discontinuous process. As sperm pass through the uterotubal junction and enter the tubal isthmus, they may be trapped and held in a reservoir. Entrapment and storage of sperm in the initial segment of the tube may serve to prevent polyspermic fertilization by allowing only a few sperm at a time to reach the oocyte in the ampulla. In addition to providing a haven, the storage reservoir maintains the fertility of sperm until ovulation. Sperm could be released from the reservoir either through loss of binding sites on the epithelium or by alterations in the sperm themselves. Sperm undergo two changes in preparation for fertilization: capacitation and hyperactivation. Capacitation involves changes in the plasma membrane, including shedding of proteins and cholesterol[39] which could reduce their affinity for the endosalpingeal epithelium. Hyperactivation, on the other hand, is a change in flagellar beating that typically involves an increase in the flagellar bend amplitude. This can provide the force necessary for overcoming the attraction between sperm and epithelium.[40] Hormonal signals that induce ovulation or signals from the pre-ovulatory follicle could stimulate the epithelium to secrete factors that trigger sperm capacitation and hyperactivation, thereby bringing about sperm release.

Hyperactivation of Sperm

Sperm becomes hyperactivated in the female tract, most likely in the Fallopian tubes. In addition to its possible role in detachment from endosalpingeal epithelium, hyperactivation is required by sperm to progress towards the oocyte in viscoelastic tubal lumen and penetrate its vestments. Hyperactivation also endows sperm with greater flexibility for turning around in pockets of

mucosa.[41,42] In the human Fallopian tube, mucosal folding increases in height and branching from the isthmus to the ampulla and thus hyperactivation may assist sperm in navigating the increasingly complex maze. In addition to assisting sperm in reaching the oocyte, hyperactivation also aids sperm in penetrating the zona pellucida.

Taxis of Sperm Towards Oocyte

Sperm taxis is defined as an oriented movement in response to a chemical or physical gradient, resulting in approaching or repelling from the attractant or repellant respectively. It is a key event in reproduction as the spermatozoa must be guided towards the eggs over long distance. There is evidence for the existence of two complementary guidance mechanisms, i.e thermotaxis (temperature dependent)[43] and chemotaxis (odorant receptor mediated)[44] operating within the fallopian tube.

Gamete Interaction and Fertilization

Capacitation

The physiological changes in sperm cells that are required for acrosome reaction and oocyte binding are collectively termed capacitation. Capacitation may enable the female tract to control the speed with which sperm gain the fertilizing capacity and thereby enable the delivery of freshly capacitated sperm to ovulated eggs. Premature acrosome reaction may lead to loss of sperm viability, therefore, capacitation and sperm transport in female tract may involve the sequestration of acrosome intact spermatozoa and their delivery to the site of fertilization in concert with ovulation. Under *in vivo* situation the capacitation occurs in female genital tract. Following are the major changes occurring in capacitating spermatozoa.

a. Cholesterol removal from sperm plasma membrane which increases the membrane fluidity[45]
b. A facilitated movement of proteins within the plasma membrane, expression of mannose binding sites and activation of progesterone receptor[46]
c. Increase in the phosphorylation of tyrosine residues in the membranes[47]
d. Influx of calcium ion from extracellular environment and subsequent increase in cAMP production[48]
e. Increase in the intracellular reactive oxygen species[49]

During capacitation, the decapacitation factors from seminal plasma which are adsorbed on the surface of the spermatozoa are removed from the sperm surface especially during their migration through cervical mucus.[50] Because of the microarchitecture of cervical mucus, the spermatozoa are squeezed through which removes the sperm coating substances.[51] The reactive oxygen species (ROS) at very low level acts as positive modulator of capacitation in female reproductive tract as well as under *in vitro* situation.[52] Although there is no definitive marker to identify the capacitation, hyperactivated motility and exocytosis of acrosomal contents may represent the end points of capacitation.

Acrosome Reaction

Upon completion of capacitation, the spermatozoa are ready to undergo acrosome reaction, which is an exocytotic event. The release and dissolution of acrosomal contents are absolutely required for the successful penetration of spermatozoa through the cumulus oophorous and zona pellucida. The major inducers of acrosome reaction are progesterone and zona protein. Follicular fluid mixed with oviductal fluid at the time of ovulation is a rich source of progesterone. Binding of spermatozoa with progesterone induces a massive calcium influx. The enzymes hyaluronidase and acrosin are released in vesicles. Hyaluronidase may help in digesting the cumulus cells and help in penetration of sperm to reach the zona.

Zona pellucida is an acellular glycoprotein coat (15 µm thick) secreted by the oocyte into the perivitelline space. It is made up of three glycoproteins- ZP3, ZP2 and ZP1. Chains of ZP2 and ZP3 are linked by ZP1. ZP2 acts as primary receptor and ZP3 acts as a secondary receptor for spermatozoa. Once the ZP3 receptor (lectin) of the sperm binds to ZP3, a further increase in intracellular calcium takes place.

The acrosin has trypsin like activity which softens the zona pellucida glycoproteins. Vigorous motility of acrosome reacted sperm and digestive activity of acrosin together may help the spermatozoa in piercing the zona to enter the perivitelline space. High intra acrosomal pH activates Phospholipase A2 enzyme which destabilizes the apposed outer acrosomal and plasma membrane ultimately causing membrane fusion by producing a lysophospholipid fusogen.[53]

Fertilization

Fertilization is the process that leads to the union of sperm and oocyte nuclei within the cytoplasm of the activated oocyte. Spermatozoa contribute to fertilization with three

important components (a) the paternal haploid genome; (b) oocyte activating factors—(c) the centrosome. The process of fertilization begins when the spermatozoa with acrosome reacted, hyperactivated spermatozoa penetrate into the outer vestments of oocyte. The hydrolytic enzymes released from acrosome, especially the hyaluronidase plays a key role in the dispersion of cumulus helping the sperm to reach till zona pellucida where it interacts with zona proteins like ZP3. The loose, sperm- ZP3 interaction further amplifies the acrosome reaction. After complete loss of acrosome, the spermatozoa lose their ZP3 receptor, however, they remain bound to another zona protein ZP2.[54] Sperm achieves zona penetration by vigorous sperm motility and enzymatic hydrolysis.

After crossing the zona, the acrosome reacted sperm reaches the perivitelline space. It binds with plasmalemma by plasma membrane at post-acrosomal region and rests tangentially with egg surface. The binding is mediated through proteins such as integrins, cadherins and fertilins.[55,56]

Sperm-egg fusion rapidly leads to activation of the oocyte, which is closely associated with abrupt increase in intracellular calcium.[57] The specific pattern of calcium oscillation in oocyte during activation is due to a protein oscillin.[58] Further entry of sperm into the perivitelline space is prevented by a complex mechanism known as cortical reaction linked oocyte activation. Rapid exocytosis of the cortical granules from the oocyte begins which brings a high concentration of proteolytic enzymes into perivitelline space. These enzymes digest the sperm binding sites (ZP3, ZP2) on the zona, and toughen and harden the zona.[59]

The oocyte activation also leads to resumption of meiosis in oocyte. The oocyte which is at metaphase II stage progresses to anaphase followed by telophase and finally completes the meiosis by dividing into two unequal cells- the fertilized oocyte and second polar body which is extruded into the perivitelline space. This event makes sure that the oocyte is in haploid state. The activation of oocyte does not necessarily from spermatozoa. Activation of oocyte without spermatozoa is known as parthenogenic activation (Example, temperature shock, pricking of the egg membrane, repeated pipetting, alcohol, etc.) which can develop up to blastocyst stage.

Pronuclei Formation and Syngamy

As the sperm head is incorporated into ooplasm, the nuclear envelope disappears and chromatin begins to decondense. The reduction of disulfide bonds by molecules like glutathione (GSH) further helps in decondensation of sperm chromatin. The sperm specific protamines are gradually replaced by the histones present in the ooplasm. A new nuclear envelope develops mostly of maternal material and a male pronucleus is thus created. Simultaneously the female pronuclei formation starts, which is poorly understood. The chromatin remaining after second polar body extrusion becomes diffuse and gets surrounded by a nuclear envelope to form female pronucleus. The fertilized oocyte with two pronuclei is termed as zygote. Active DNA synthesis starts in both male and female pronuclei. Sperm aster—a new microtubule formed structure derived from centrosome of the spermatozoa assembles in the ooplasm.[60] The radially arrayed sperm aster is adjacent and affixed to the sperm nucleus. It helps in pushing the two pronuclei until they appose in the center of the oocyte. This event is followed by the breakdown of the nuclear envelope and fusion of the pronuclei. Simultaneously the centrosome duplicates and separates, originating the two poles of the first mitotic spindle. The chromosomes from male and female gametes are released into the ooplasm and get mixed as they align at metaphase plate of the first mitotic spindle. At this stage the first cleavage division begins resulting in production of zygotic nucleus that contains chromosomes of maternal and paternal origin. Duration of the full mitotic cycle is 20-22 hr but each zygote may show a marked difference in length of the each phase.

REFERENCES

1. Turner TT, Gleavy JL and Harris JM. Fluid movement in the lumen of the rat epididymis: effect of vasectomy and subsequent vasovasostomy. J Androl 1990;l1:422-28.
2. Robaire B and Hermo L. Efferent ducts, epididymis, and vas deferens: structure, function and their regulation. In: Knobil E, Neil J, eds. The Physiology of Reproduction. New York: Raven Press 1988:999-1080.
3. Wong PYD, Au CL, Nagi HK. Electrolyte and water transport in rat epididymis: its possible role in sperm maturation. Int J Androl 1978;(Suppl) 2:608-28.
4. Wong PYD, Yeung CH. Hormonal control of fluid reabsorption in isolated rat cauda epididymis. Endocrinology 1977;l0l:1371-97.

5. Turner TT, Cesarini DM. The ability of the rat epididymis to concentrate spermatozoa. J Androl 1983;3:l97-202.
6. Hinton BT, Keefer DA. Binding of 3H-aldosterone to a single population of cells within the rat epididymis. J Steroid Biochem 1985;23:231-33.
7. Wong PYD, Uchendu CN. The role of angiotensin-converting enzyme in the rat epididymis. Endocrinol 1990;125:457-65.
8. Moore HDM, Hartman TD, Pryor JP. Development of the oocyte-penetrating-capacity of spermatozoa in the human epididymis. Int J Androl 1983;6:310-18.
9. Janulis LJ, Hess RA, Bunick D, Nitta H, Janssen S, Asawa Y, Bahr JM. Mouse epididymal sperm contain active P450 aromatase which decreases as sperm traverse the epididymis. J Androl 1996;17, 111-16.
10. Yeung CH, Sonnenberg-Riethmacher E, Cooper TG. Infertile spermatozoa of c-ros tyrosine kinase receptor knockout mice show flagellar angulation and maturational defects in cell volume regulatory mechanisms. Biol Reprod 1999;61:1062-69.
11. Aitken RJ, Krausz C. Oxidative stress, DNA damage and Y chromosome. Reproduction 2001;122:497-506.
12. Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, Van Zyl JA, Smith K. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril 1986;46:1118-23.
13. Sarkar S, Jones OW, Shioura N. Constancy in human sperm DNA content. Proc Natl Acad Sci USA 1974;71:3512-16.
14. World Health Organization (WHO) laboratory manual. 4th ed. Cambridge, UK: Cambridge University Press 1999.
15. Menge AC, Edwards RP. Mucosal immunity of the reproductive tract and infertility. In Zaz RK (Ed.), Immunology of Reproduction. CRC Press, Boca Raton, FL 1993:19-36.
16. Lilja H, Lundwall A. Molecular cloning of epididymal and seminal vesicular transcripts encoding a semenogelin-related protein. Proc Natl Acad Sci USA 1992;89:4559-63.
17. Harper MJK. Gamete and zygote transport. In Nobil E and Neill JD (Eds), The Physiology of Reproduction, 2nd edn. Raven Press Ltd., New York, NY 1994:123-87.
18. Lundwall A, Giwercman A, Ruhayel Y, Giwercman Y, Lilja H, Hallden C, Malm J. A frequent allele codes for a truncated variant of semenogelin I, the major protein component of human semen coagulum. Mol Hum Reprod 2003;9:345-50.
19. Lilja H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J Clin Invest 1985;76:1899-903.
20. Baker RR, Bellis MA. Human sperm competition: Ejaculate manipulationby females and a function for the female orgasm. Anim Behav 1993;6:887-909.
21. Watt KW, Lee PJ, M' Timkulu T, Chan WP, Loor R. Human prostate-specific antigen: structural and functional similarity with serine proteases. Proc Natl Acad Sci USA 1986;10:3166-70.
22. Sobrero AJ, MacLeod J. The immediate postcoital test. Fertil Steril 1962;13:184-89.
23. Dostal J, Veselsky L, Marounek M, Zelezna B, Jonakova V. Inhibition of bacterial and boar epididymal sperm immunogenicity by boar seminal immunosuppressive component in mice. J Reprod Fertil 1997;111:135-41.
24. Katz DF, Slade DA, Nakajima ST. Analysis of preovulatory changes in cervical mucus hydration and sperm penetrability. Adv Contracep 1997;13:143-51.
25. Carlstedt I, Sheehan JK. Macromolecular architecture and hydrodynamic properties of human cervical mucins. Biorheology 1984;21:225-33.
26. Katz DF, Mills RN, Pritchett TR. The movement of human spermatozoa in cervical mucus. J Reprod Fertil 1978;53:259-65.
27. Gould JE, Overstreet JW, Hanson FW. Assessment of human sperm function after recovery from the female reproductive tract. Biol Reprod 1984;31:888-94.
28. Mortimer D. Practical Laboratory Andrology. Oxford University Press, New York, NY 1994.
29. Katz DF, Morales P, Samuels SJ, Overstreet JW. Mechanisms of filtration of morphologically abnormal human sperm by cervical mucus. Fertil Steril 1990;54:513-16.
30. Yudin AI, Hanson FW, Katz DF. Human cervical mucus and its interaction with sperm: a fine-structural view. Biol Reprod 1989;40:661-71.
31. Pandya IJ, Cohen J. The leukocytic reaction of the human uterine cervix to spermatozoa. Fertil Steril 1985;43:417-21.
32. Parkhurst MR, Saltzman WM. Leukocytes migrate through three-dimensional gels of midcycle cervical mucus. Cell Immunol 1994;156:77–94.
33. Matthur S, Rosenlund C, Carlton M, Caldwell J, Barber M, Rust PF and Williamson HO. Studies on sperm survival and motility in the presence of cytotoxic sperm antibodies. Am J Reprod Immunol Microbiol 1988;17:41-47.
34. Jensen TS, Bjorge L, Wollen AL, Ulstein M. Identification of the complement regulatory proteins CD46, CD55, and CD59 in human fallopian tube, endometrium, and cervical mucosa and secretion. Am J Reprod Immunol 1995;34:1-9.
35. Ulcova-Gallova Z. Ten-year experience with antispermatozool activity in ovulatory cervical mucus and local hydrocortisone treatment. Am J Reprod Immunol 1997;38:231-34.
36. Mortimer ST, Swan MA. Variable kinematics of capacitating human spermatozoa. Hum Reprod 1995;10:3178-82.
37. Kunz G, Beil D, Deininger H, Wildt L, Leyendecker G. The dynamics of rapid sperm transport through the female genital tract: Evidence from vaginal sonography of uterine peristalsis and hysterosalpingoscintigraphy. Hum Reprod 1996;11:627-32.

38. Jansen RPS. Cyclic changes on the human fallopian tubes isthmus and their functional importance. Am J Obstet Gynecol 1980;136:292-308.
39. De Jonge C. Biological basis for human capacitation. Hum Reprod Update 2005;11:205-14.
40. Ho HC, Suarez SS. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction 2001;122:519-26.
41. Suarez SS, Katz DF, Overstreet JW. Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. Biol Reprod 1983;29:1277-87.
42. Suarez SS, Osman RA. Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biol Reprod 1987;36:1191-98.
43. Bahat A, Tur-Kaspa I, Gakamsky A, Giojalas LC, Breitbart H, Eisenbach M. Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. Nat Med 2003;9:149-50.
44. Spehr M, Gisselmann G, Poplawski A, Riffell JA, Wetzel CH, Zimmer RK, Hatt H. Identification of a testicular odorant receptor mediating human sperm chemotaxis. Science 2003;299:2054-58.
45. O'Rand MG. Modification of the sperm membrane during capacitation. Ann N Y Acad Sci 1982;383:392-404.
46. Benoff S. Carbohydrates and fertilization: an overview. Mol Hum Reprod 1997;3:599-637.
47. Carrera A, Moos J, Ning XP, Gerton GL, Tesarik J, Kopf GS, Moss SB. Regulation of protein tyrosine phosphorylation in human sperm by calcium/calmodulin dependent mechanism: identification of A kinase anchor proteins as a major substrates for tyrosine phosphorylation. Dev Biol 1996;180:284-96.
48. Baldi E, Casano R, Flasetti C, Krausz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl 1991;12:323-30.
49. de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997;3:175-94.
50. Kanwar KC, Yanagimachi R, Lopata A. Effects of human seminal plasma on fertilizing capacity of human spermatozoa. Fertil Steril 1979;31:321-27.
51. Overstreet JW, Katz DF, Yudin AI. Cervical mucus and sperm transport in reproduction. Semin Perinatol 1991;15:149-55.
52. Langlais J, Roberts K. A molecular membrane model of sperm capacitation and the acrosome reaction of the mammalian spermatozoa. Gamete Res 1985;12:183-224.
53. O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: Triggering and modulation of phosphorylation events. Free Radic Biol Med 2006;41:528-40.
54. Bleil JD, Greve JM, Wassarman PM. Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. Dev Biol 1988;128:376-85.
55. Bigler D, Chen M, Waters S, White JM. A model for sperm-egg binding and fusion based on ADAMs and integrins. Trends Cell Biol 1997;7:220-25.
56. Primakoff P, Myles DG. The ADAM gene family: Surface proteins with adhesion and protease activity. Trends Genet 2000;16:83-87.
57. Schultz RM, Kopf GS. Molecular basis of mammalian egg activation. Curr Topics Dev Biol 1995;30:21-62.
58. Parrington J, Swann K, Shevchenko VI, Sesay AK, Lai FA. Calcium oscillations in mammalian eggs triggered by a soluble sperm factor. Nature 1999;379:364-68.
59. Yanagimachi R. Mammalian fertilization. In Knobil E, Neil JD, eds. The Physiology of Reproduction. New York: Raven Press 1994:189-317.
60. Schatten G. The centrosome and its mode of inheritance: The reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev Biol 1994;165:299-335.

CHAPTER 3

Understanding the Physiology of Fertilization

Ashok Agarwal, Andrew Luu

ABSTRACT

The growing trend of male factor infertility incidences, along with a decrease in sperm quality over recent years, is a cause for concern. Intracytoplasmic sperm injection (ICSI) and other assisted reproductive technologies have provided patients with an alternative to their infertility woes by mimicking natural fertilization in the laboratory.

However, because ICSI bypasses the initial pathological cause for infertility, possible transmission of genetic defects can occur. In addition, a relatively low live-birth rate is still associated with ART. Further development in the understanding of gamete maturation and fertilization still needs to be elucidated. In order to optimize the outcomes of ART, all aspects of natural reproduction must be understood, including fertilization, gamete maturation and embryonic development.

INTRODUCTION

The combination of the haploid chromatin content from the maternal and paternal gamete is the most essential event in human reproduction. However, completely understanding fertilization is perhaps one of the most difficult things to accomplish. Developed in 1779 by Lazzaro Spallanzani, the notion of two gametes fusing into one conglomerate and then subsequently becoming a brand new organism is both mind-boggling and captivating. After years of evolution, the specialization of these two gametes is complex with so many intricacies that it is difficult to grasp them all. The machinery and processes that these gametes undergo prepare them for maturation, fertilization, and embryonic development. Each carries half of the genetic material for a complete embryo, and each provides materials to sustain the developing cells.

Evaluation of the gametes and their preparation for fertilization will aide clinicians and researchers in recreating fertilization *in vitro* for assisted reproductive technologies. Once differentiated into a specialized cell, the male gamete and female gamete undergo a series of events before they gain the capability to unite with each other. After fertilization, the zygote proliferates and implants onto the endometrium of the uterus, where it will develop into a fetus.

Regarding infertility, the significance in attempting to understand fertilization lies in the increasing use of Assisted Reproductive Technologies (ART). The trend correlates to the recurring incidences of infertility. When infertility is present in 15-20% of reproductive age couples, it becomes a major concern. ART provides patients with an alternative to their infertility woes, but with only a 35% live birth rate from ART procedures, ART is far from a success. Furthermore, studies have shown that gamete quality has decreased over the past few years due to both environmental factors and genetic factors; thereby, reiterating the importance of obtaining optimal efficiency in fertilization.

THE MALE GAMETE

Sperm dysfunction affects approximately 1 in 15 men and is the prevalent cause for male factor infertility; therefore, understanding the function of sperm and how they are generated is essential. The role of sperm is to bring the haploid genome to the oocyte, bind to the zona pellucida, and fuse with the oocyte. Through a process termed spermatogenesis, the spermatozoa are generated from totipotent stem cells within the testes. The two testes can

produce more than 1000 spermatozoa per second. Spermiogenesis is the final stage of spermatogenesis and transforms the round spermatids into spermatozoan. The well-organized epithelium of the testes and the somatic Sertoli cells assist in the control of spermatogenesis rate.

Endocrine factors play a significant role in regulating the proliferation of spermatozoa. The hypothalamic-pituitary-gonadal axis produces these endocrine factors. The follicle stimulating hormone (FSH) and luteinizing hormone (LH), secreted by the pituitary gland, affect the rate of spermatogenesis by activating the testicular Leydig cells and Sertoli cells. The Sertoli cells are the main target for hormonal control, because glial cell line-derived neurotrophic factor (GDNF), which is secreted by the Sertoli cells, mediates the transition between spermatogonia and spermatocytes.

The Journey: The sperm have to migrate through the rigorous environment of the female reproductive tract, which acts both selectively and positively towards the millions of sperm, depending on their morphological and functional integrity (Figure 3.1). Once formed, the spermatozoa are immotile when they are released into

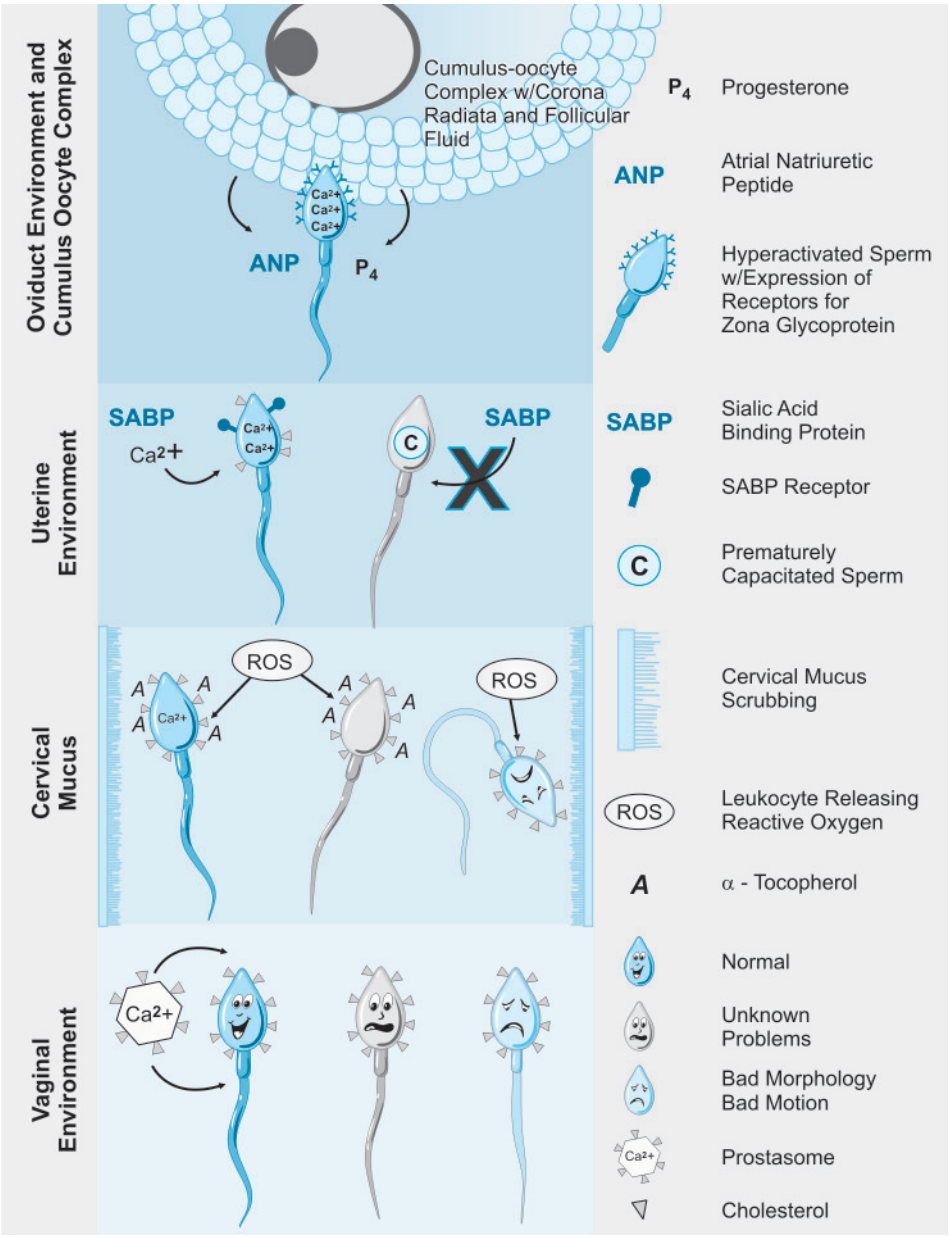


Figure 3.1: The journey of the sperm through the selective female reproductive system

the luminal fluid and transported to the retes testes. From the retes testes, it takes the spermatozoa two to six days to be passively moved to the epididymis, where they are stored until ejaculation. Upon ejaculation, the sperm accumulate secretions from the accessory sex glands and are deposited into the vaginal canal. Within the secretions are calcium-storing, cholesterol-enriched vesicles called prostasomes (Arienti et al 2004). The alkaline pH of the secretions protects the sperm from the acidic conditions of the cervical environment for a short period of time during the transport through the vaginal walls and the cervical canal. The proportion of sperm that actually enters the cervical mucus is unknown. The cervical crypts in the cervix secrete a mucus that may contain antibodies that prevent the sperm from entering the uterine cavity. Sperm concentration, motility, and morphology have been shown to affect migration within the canal. Evidence suggests that α-tocopherol and reactive oxygen species "ROS" can improve motility parameters and initiate capacitation in mammalian sperm (Agarwal et al 2003; Breininger et al 2005). On the other hand, ROS in above-physiological amounts can have detrimental effects on both DNA integrity and sperm motility parameters (Agarwal et al 2007).

Human sperm receive a lot of guidance from the female reproductive system. Within the uterine environment, sialic acid binding protein (SABP), secreted by the endometrial walls, is found to bind to sperm and helps the influx of calcium during sperm capacitation (Banerjee and Chowdhury, 1995). Atrial natriuretic peptides (ANPs) in the follicular fluid have been shown to affect sperm swimming speed and act as a chemoattractant towards sperm *in vitro* (Zamir et al 1993; Anderson et al 1995). The female reproductive system aids in modulating sperm progression and regulates the number of sperm that enter the ampulla of oviduct, which is the site of fertilization. In addition, evidence indicates that a higher percentage of sperm undergo the acrosome reaction when exposed to progesterone.

THE FEMALE GAMETE

Some of the more important structures of the oocyte, regarding fertilization, are the zona pellucida (ZP) and polar bodies. The oocyte is responsible for a few more functions as well, such as nourishing the embryo, providing structural orientation and protecting the embryo. The egg shell surrounding the oocyte consists of two layers, which coat the vitellus and the vitelline membrane—the outer cumulus oophorus and the inner zona pellucida. The cumulus oophorus consists of mainly a matrix of granulose cells and hyaluronic acid, while the zona pellucida consists of glycoproteins. The ZP mediates sperm-egg recognition and induces the acrosome reaction in the sperm. The ZP is also responsible for preventing polyspermy and enveloping the embryo until implantation. The polar bodies, on the other hand, are responsible for the asynchronous division during the first meiotic cycle. The morphology of the first polar body is related to oocyte quality.

Oogenesis is the process by which mature oocytes are generated. Some differences from spermatogenesis are that oocytes are not derived from totipotent stem cells and that there are two arrest phases in the process. The preparation of the oocyte for fertilization is essential, because if any of the events (ZP acquisition, cortical granule formation, protein synthesis, cytoskeletal changes, etc.) are interrupted, development will be abnormal. Similar to spermatogenesis, oogenesis is controlled by endocrine factors as well. Estrogen, progesterone, LH and FSH levels are responsible for the follicular, ovulation, and luteal phases of oocyte maturation.

THE JOURNEY: The fallopian tubes are the mode of transport for oocytes. Surge in LH induces completion of first meiotic division and continues until arrest at metaphase II. Ovulation yields the oocyte-cumulus complex, which consists of the ZP and granulose cells (cumulus oophorus). The complex is then transported to the ampulla via the ciliated surface within the infundibulum of the oviduct.

Physiology of Fertilization

Sperm preparation is the first step in initiating fertilization, because the spermatozoa are not fully functional after being deposited into the female reproductive tract (i.e. no ability to penetrate the oocyte membranes). The maturation process occurs on the journey towards the oocyte, and the events are termed capacitation. Capacitation allows the sperm to penetrate the barriers of the oocyte, fuse with the oolemma of the oocyte, and exocytose relevant contents. Initiation of capacitation depends on a variety of mechanisms (Figure 3.2), such as ion influxes that cause changes in the membrane potential and the removal of cholesterol

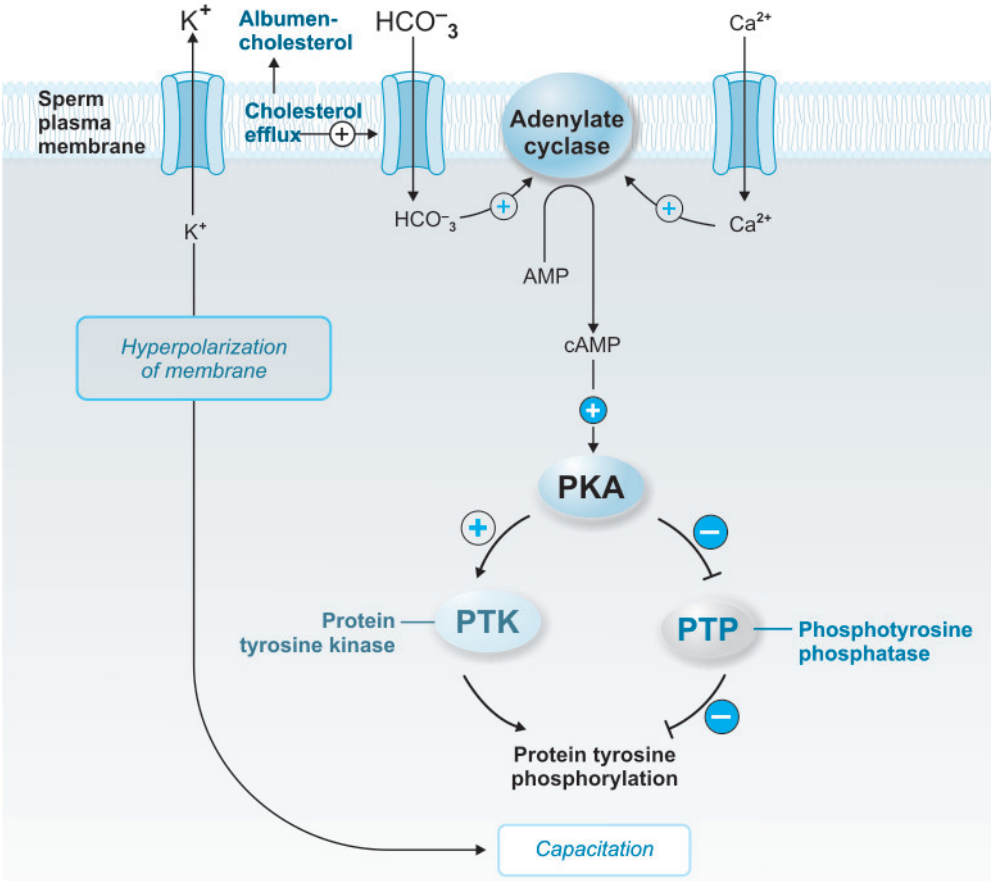


Figure 3.2: Summary of two possible pathways for the activation of sperm capacitation- 1) influx of calcium (Ca^{2+}) or 2) the hyperpolarization of the membrane via K^+ channel activation. Both processes have been suggested to be ZP3 receptor regulated

from the sperm plasma membrane. Two events occur after initiation- hyperactivation in the tail and acrosome reaction in the head. Hyperactivation increases the speed, velocity and beat frequency of the tail, allowing the sperm to penetrate the cumulus oophorus and ZP of the oocyte. Hindered hyperactivation does not bode well for *in vitro* fertilization procedures (IVF). Acrosome reaction results in morphological changes of the acrosome and is triggered by an extracellular calcium influx into the sperm cell or intracellular second messengers. The hydrolytic enzymes within the acrosome (most studied are hyaluronidase and acrosin) are responsible for digesting the extracellular matrix of the oocyte. The acrosome reaction also aids in recognition, adhesion and fusion with the oocyte.

The next step in fertilization is the incorporation of the sperm into the egg cytoplasm. The three ZP glycoproteins have been shown to aid in sperm reception, and the cumulus oophorus has been demonstrated to aid in initiating the acrosome reaction.

Oocyte activation is considered the third step in fertilization and is initiated by sperm entry. The release of cortical granules, activation of membrane bound adenosine triphosphatases (ATPases), and resumption of meiosis are all events that indicate oocyte activation. One of the essential events during activation is the release of cortical granules to block polyspermy. The vitelline membrane hardens and receptors are removed on the membrane.

The fourth step of fertilization is the formation of the sperm and oocyte pronuclei. Mitochondria are activated, and the centrosome aids in the formation of asters next to the sperm nucleus. Glutathione, which is produced from the oocytes, aids in the formation of the male pronucleus by remodeling the sperm chromatin. Genomic union of the pronuclei follows the formation of

the gamete pronuclei. The centriole, which is derived from the sperm, acts as a microtubule organizing center that unites the male and female pronuclei. The continuing elongation of the microtubules allows the microtubules to come into contact with the oocyte pronucleus and transport it to the region next to the male pronucleus. The eventual union of the pronuclei results in centrosome degradation.

The final step of fertilization is the initiation of the first division and early development. After union of the pronuclei, the nuclear membrane dissolves, and DNA undergoes replication. The accessory structures of the sperm are also dissolved and removed. The events result in the first mitotic division to form a 2-cell embryo.

CAPACITATION – IN DEPTH

Sperm capacitation is a complex series of events that prepares the sperm to bind and fuse with an oocyte once it is deposited into the female genital tract. As mentioned previously, capacitation involves both the acrosome reaction and hyperactivation. The molecular basis for these events is not clear, but many studies have indicated that removal of cholesterol from the plasma membrane, increases in pH and calcium (Ca^{2+}) levels, and activation of second-messenger cascades could be responsible for capacitation (de Lamirande et al 1997, Purohit et al 1999, Darszon et al 2001). The increased levels of intracellular calcium could be due to the inhibition of the Ca^{2+} ATPase pump causing reduced Ca^{2+} efflux, the instability of the membrane from removal of cholesterol, and the activation of unknown channels that cause a Ca^{2+} influx. Other evidence supports these notions and suggests that specific T-type voltage-gated calcium channels (VGCCs) might play a role in setting the Ca^{2+} levels. Serum albumin expands the Ca^{2+} current by altering the voltage dependencies of activation and inactivation in the VGCCs (Espinosa et al 2000), possibly facilitating an increase in Ca^{2+} influx. The regulation of these VGCCs is correlated with tyrosine phosphorylation (Visconti and Kopf 1998, Ficarro et al 2003).

In addition to Ca^{2+}, increases in pH have been correlated with capacitation (de Lamirande et al 1997, Purohit et al 1999). Induced hyperpolarization of the membrane potential can be observed during capacitation, and suggestions that Ca^{2+} or pH regulated potassium (K^+) channels play a role in causing the hyperpolarization have been observed (Arnoult et al 1997; Felix et al 2005). This hyperpolarization is considered the initiator that activates the T-type VGCCs to respond to stimuli from the ZP (Arnoult et al 1997). Some pH-dependent K^+ channels that are inward rectifiers have been discovered in spermatogenic cells (Munoz-Garay et al. 2001). The activation of these channels result in an increased K^+ permeability and hyperpolarization of the membrane as well.

Regarding the acrosome reaction (AR) during capacitation, exocytosis of the acrosomal vesicle via ZP3-mediated events yields proteolytic enzymes that are responsible for gamete fusion. Ca^{2+} influx is the requirement for this physiological response (Publicover and Barratt 1999, Darszon et al 2001), and hyperactivation and hyaluronidase allow for the penetration of the cumulus cells of the oocyte (Primakoff and Myles 2002). The increased Ca^{2+} levels and exocytosis via ZP3 mediated events can proceed through two pathways—activation of guanosine triphosphate (GTP)-binding protein and phospholipase C mechanisms or activation of VGCC. The sustained increase in Ca^{2+} influx is maintained by transient receptor potential cation (TRPC) family Ca^{2+} channels, thus, triggering exocytosis (Darszon et al 2001, Primakoff and Myles 2002).

EMBRYONIC DEVELOPMENT

Three developmental stages are commonly associated with prenatal ontogenetic development: the germinal stage, the embryonic development stage, and the fetal development stage. The germinal stage starts at the fertilization of the ovum and proceeds through blastogenesis, allowing for the formation of a transitory trophectoderm, the trophoblast. The outer cell layer of the extraembryonic trophoblast is responsible for the successful implantation of the blastocyst onto the uterine wall and the formation of a placenta. The blastocyst contains an inner cell mass, which gives rise to the early embryo. The placenta is essential because the human egg is void of yolk, so nutrition and oxygen are supplied via the placenta during embryonic development.

Most successful pregnancies result when implantation occurs 7 to 10 days after ovulation. Successful implantation requires multiple factors, including a receptive endometrium, normal blastocyst functionality and the formation of a placenta. Removal of the ZP with the help of *strypsin* results in the "hatching" of the blastocyst from the ZP. The window of opportunity for

implantation and endometrial receptivity is quite small and is marked by the appearance of pinopodes (membrane projections).

Gastrulation immediately follows successful implantation and is considered as the first phase of embryogenesis. Three germinal layers are formed: ectoderm, mesoderm and entoderm. Once the layers are spatially separated, neurulation begins. During neurulation, each layer subdivides into specific organ precursors, and then eventually, organogenesis occurs. The ectoderm gives rise to the epidermal ectoderm and the neural ectoderm. The mesoderm gives rise to the ectomesenchyme, which consists of the meninges and the spinal ganglia, and the entomesoderm, which ultimately leads to the formation of numerous diverse tissues, including skeletal muscles, kidneys, spine, and blood vessels. The entoderm gives rise to the intestinal epitheliums and intestinal derivatives. Embryogenesis encompasses the above processes and results in the initial morphology of the organs within the human embryo. The timeframe for which embryogenesis occurs is considered too last up to the 8th week of gestation. The time period after the 10th week and up onto birth is considered fetal development. Fetal development is responsible for the primary growth of the organism. In addition, final differentiation of all the organs occurs during fetal development (Larsen et al 2001).

Activation of Embryonic Genome: All mitotically active cells have nucleoli, which are located within the nuclei, and are considered the protein powerhouses of the cell. Nucleoli develop on the rRNA coding sites of the chromosomes. They are first seen in oocytes, because the exponential growth of the oocyte demands a high rate of protein synthesis. Full development and activation of the nucleoli continues through the first few mitotic cycles of the embryo, until it obtains full functionality at the activation of the embryonic genome.

PATHOPHYSIOLOGY OF FERTILIZATION AND THE MALE GAMETE

Interruption of fertilization can occur at any level, from gamete formation to postfertilization development. DNA damage to the genes that control the molecular mechanisms in gamete formation subsequently affects the fertilization potential. Sperm DNA damage has been demonstrated to affect the motility and morphology of the sperm and lower its functionality. The structure of the spermatozoa allows it to be quite susceptible to exogenous factors also. Having polyunsaturated fatty acids (PUFAs) as the main component in the cellular membrane is somewhat disadvantageous for the sperm. The PUFAs have higher energy bonds, so they are less stable and more likely to react with surrounding molecules. Studies have demonstrated that lower pregnancy rates and lower semen parameters are correlated with higher levels of DNA fragmentation (Benchaib et al 2003, Moskovtsev et al 2007). Sources of DNA damage range from exogenous radiation to oxidative stress caused by an increased level of reactive oxygen species (ROS; Figure 3.1). Sources of ROS include leukocytes, cellular metabolism, and exogenous introduction. Once ROS levels surpass the physiological defense levels, lipid peroxidation and apoptosis can be induced.

Interruption of the hormonal pathways that control both gamete production and embryonic development would also reduce fertilization success. On the other hand, fertilization might occur, but development would fail. Endocrine disruptors, such as vinclozolin and diethylstilbestrol (DES), have been shown to increase the rate of genital malformations, cryptorchidism, and testicular cancer (Strohsnitter et al 2001). In a study by Anway et al (2005), exposure to vinclozolin led to higher rates of apoptosis, lower rates of spermatogenesis, and lower sperm motility parameters. Endocrine disruptors can have detrimental effects to the fertilization potential of the male gamete and to the male reproductive organs. Furthermore, the composition of the male gamete can be compromised by protamine levels. Irregular ratios between the two protamines present in humans have been correlated with increased DNA damage and decreased viability (Aoki et al 2006).

Aside from the gamete level, the preimplanted embryo is also susceptible to environmental factors (Varghese et al 2008). Uterine receptivity for implantation is currently considered one of the major limiting factors for successful pregnancies. In addition, diseases of the reproductive organs and tracts can have detrimental effects on gamete quality and fertilization potential as well (i.e. urinary tract infections, sexually transmitted diseases, and varicoceles).

Assisted Reproductive Technology to Aid in Fertilization

With the growing trend of male factor infertility (Varghese et al 2008) and the desire for infertile couples to reproduce, assisted reproductive technology provides

a means to bypass their woes. Several techniques exist depending on the extent of the infertility. Intrauterine insemination (IUI) is used when there is only slight oligospermia and involves artificial insemination after induced ovulation. For cases of severe oligospermia or even azoospermia, a combination of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) is used. IVF attempts to imitate fertilization outside of the human body, by placing sperm in the vicinity of the oocyte and allowing fertilization to proceed with the help of various reagents. ICSI, on the other hand, involves mechanical breeching of the oocyte and injection of a single spermatozoan into the egg cytoplasm. In conjunction with ICSI, the development of testicular sperm extraction (TESE) allows patients with idiopathic azoospermia or retrograde ejaculation to induce pregnancy. Moreover, when motile sperm are obtained from testicular tissue, they are functionally comparable to ejaculated spermatozoa. ICSI also decreases the necessary number of oocytes during oocyte retrieval.

ICSI results are dependent on a few factors: spermatozoal factors, female factors, oocyte activation, and oocyte injury as a result of cytoplasmic injection. Studies such as Nagy et al (1998) discovered that motility is a significant predictor of pregnancy success and suggested that motility is a good indicator for viability as well. Tail function, another spermatozoal factor, has been shown to affect fertilization rate as well. Aggressive immobilization of spermatozoa through tail manipulation has been used to increase sperm membrane permeability. Gerris et al (1995) compared fertilization rates achieved using sperm with intact tails compared to sperm with damaged tails during ICSI. Aggressive immobilization of spermatozoa resulted in an increase of 36 to 60% in fertilization rate. The authors suggested that tail damage induces sperm membrane changes which facilitate biochemical events necessary for pronuclear formation. Regarding female factors, Spandorfer et al (1998) discovered that pregnancy rates were significantly lower with increased maternal age, even though fertilization rates were unaffected. Similar results were found by Spandorfer et al (2004), with an increase in the rate of aneuploidy for embryos derived from the oocytes of women over 40 compared to those from women less than age 35. Therefore, fertilization rates may be less dependent on female factors. Overall ICSI success, however, has been shown to be strongly correlated to oocyte quality.

ICSI can also depend on oocyte activation, because it is an essential event during fertilization. Oocyte activation may not necessarily occur during ICSI. Tesarik and Sousa (1995) demonstrated that intentional induction of oocyte activation during ICSI, with aggressive aspiration and injection of the oocyte cytoplasm, yielded higher fertilization and pregnancy rates. Intracellular calcium changes have been shown to play a role in oocyte activation, and the aggressive aspiration of the oocyte cytoplasm led to spikes in intracellular calcium levels. Induction of calcium ionophores and oscillin has been used for fluctuations in oocyte calcium levels.

Cytoplasmic injury in the oocyte is another factor that can alter the results of ICSI. Although the precise reasons for oocyte injury are unknown, it is predicted to be a result of plasma membrane and ultrastructural disturbances associated with the injection of the spermatozoan and/or extrusion of the oocyte cytoplasm following injection. Technique plays a large role in rate of oocyte injury as well. As greater expertise is gained, oocyte injury rate may decrease.

When comparing ICSI with other ART procedures, ICSI has a relatively lesser amount of limiting factors, because it requires the least amount of available sperm and can utilize immature and immotile sperm for the procedure. A study by Lie et al (2005) denounced the idea that there is an increased risk in ICSI babies when compared to IVF. However, as promising as ICSI appears to be, clinicians are ultimately bypassing the pathological role that made the men infertile in the first place. This could lead to the transmission of genetic defects. In addition, immature sperm, such as round spermatids, extracted from testicular tissue yielded poor results in ICSI procedures. Lower fertilization rates and slower embryonic development were observed when using round spermatids (Levran et al 2000).

When compared to natural fertilization, a review by the ESHRE Capri Workshop (2007) found that ICSI fetuses had a significantly higher number of *de novo*, sex chromosome, and inherited chromosomal abnormalities in certain experiments. Another study also found that paternal structural chromosomal anomalies were responsible for inherited abnormalities (Bonduelle et al 2002).

CONCLUSION

Fertilization and embryonic development are intricate processes that involve the fusion of two highly specialized gametes. The mechanism of genesis for the two gametes is also both unique and complex. Because of the manner in which the mechanisms encompass multiple systems, the susceptibility to deleterious effects is present, thus, leading to infertility. Elucidating how and why a particular sperm is more adept than others at reaching the oocyte and penetrating it, or a particular embryo is more capable of surviving, will allow researchers to develop a better embryo selection method. Perhaps with current methods, the *in vitro* culture system is only selective for embryos that can develop in that specific system.

Progress in medicine can only be achieved if efforts are made for a better understanding of the etiology of male infertility. While some experiments link ICSI to poor outcomes for babies, it is still not clear whether ART or infertility is to be blamed. Future development should proceed in areas on blastocyst transfer, maximizing the implantation window, better sperm selection methods, and understanding the role of capacitation in ART success.

BIBLIOGRAPHY

1. Agarwal A, Prabakaran SA, Sikka SC. Clinical Relevance of Oxidative Stress in Patients with male Factor Infertility: Evidence Based Analysis. In: AUA Update Series 2007;1-11.
2. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 2003;79(4):829-43.
3. Anderson RA, Jr, Feathergill KA, Rawlins RG, Mack SR, Zaneveld LJ. Atrial natriuretic peptide: A chemoattractant of human spermatozoa by a guanylate cyclase-dependent pathway. Mol Reprod Dev 1995;40(3):371-78.
4. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005;308(5727):1466-69.
5. Aoki VW, Emery BR, Liu L, Carrell DT. Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. J Androl 2006;27(6):890-98.
6. Arienti G, Carlini E, Saccardi C, Palmerini CA. Role of human prostasomes in the activation of spermatozoa. J Cell Mol Med 2004;8(1):77-84.
7. Arnoult C, Lemos JR, Florman HM. Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. Embo J 1997;16(7):1593-99.
8. Baarends WM, Grootegoed JA. Male gametogenesis. In: Fauser BCJM, Bouchard P, Hsueh AJW et al (Eds). Reproductive Medicine: Molecular, Cellular and Genetic Fundamentals. 2nd ed: Informa Healthcare 2002;381-400.
9. Banerjee M, Chowdhury M. Purification and characterization of a sperm-binding glycoprotein from human endometrium. Hum Reprod 1994;9(8):1497-1504.
10. Benchaib M, Braun V, Lornage J, et al. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 2003;18(5):1023-28.
11. 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):2600-14.
12. Breininger E, Beorlegui NB, O'Flaherty CM, Beconi MT. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. Theriogenology 2005;63(8):2126-35.
13. Darszon A, Beltran C, Felix R, Nishigaki T, Trevino CL. Ion transport in sperm signaling. Dev Biol 2001;240(1):1-14.
14. de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997;3(3):175-94.
15. De Sutter P, Van der Elst J, Coetsier T, Dhont M. Single embryo transfer and multiple pregnancy rate reduction in IVF/ICSI: A 5-year appraisal. Reprod Biomed Online 2003;6(4):464-69.
16. Dean J. Oocyte-specific gene expression: Roles in folliculogenesis, fertilization, and early development. In: Trounson AO, Gosden RG, (Eds). Biology and Pathology of the Oocyte: Its Role in Fertility and Reproductive Medicine. 1st ed: Cambridge University Press; 2003: 67-80.
17. Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. Hum Reprod Update 2004;10(1):19-28.
18. Eisenbach M, Giojalas LC. Sperm guidance in mammals-an unpaved road to the egg. Nat Rev Mol Cell Biol 2006;7(4):276-85.
19. Esfandiari N. Normal Fertilization and Implantation. In: Falcone T, Hurd WW, editors. Clinical Reproductive Medicine and Surgery: Mosby. 2007;101-14.
20. Espinosa F, Lopez-Gonzalez I, Munoz-Garay C, et al. Dual regulation of the T-type Ca^{2+} current by serum albumin and beta-estradiol in mammalian spermatogenic cells. FEBS Lett 2000;475(3):251-56.
21. Felix R. Molecular physiology and pathology of Ca^{2+}: Conducting channels in the plasma membrane of mammalian sperm. Reproduction. 2005;129(3):251-62.
22. Ficarro S, Chertihin O, Westbrook VA, et al. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. J Biol Chem 2003;278(13):11579-89.

23. Gerris J, Mangelschots K, Van Royen E, Joostens M, Eestermans W, Ryckaert G. ICSI and severe male-factor infertility: breaking the sperm tail prior to injection. Hum Reprod 1995;10(3):484-86.
24. Hewitson L, Ramalho-Santos J, Simerly C, Schatten G. Fertilization: Fate of sperm components after ICSI. In: Trounson AO, Gosden RG, (Eds). Biology and Pathology of the Oocyte: Its Role in Fertility and Reproductive Medicine. 1st ed: Cambridge University Press; 2003: 133-40.
25. Hewitson L, Simerly C, Schatten G. Fertilization. In: Fauser BCJM, Bouchard P, Hsueh AJW et al., editors. Reproductive Medicine: Molecular, Cellular and Genetic Fundamentals. 2nd ed: Informa Healthcare; 2002;401-20.
26. Intracytoplasmic sperm injection (ICSI) in 2006: evidence and evolution. Hum Reprod Update 2007;13(6):515-26.
27. Jagannathan S, Publicover SJ, Barratt CL. Voltage-operated calcium channels in male germ cells. Reproduction 2002;123(2):203-15.
28. Kalthoff KO. Gametogenesis. In: Kalthoff KO, editor. Analysis of Biological Development. 2nd ed: McGraw-Hill Science/Engineering/Math; 2000.
29. Kalthoff KO. Fertilization. In: Kalthoff KO, editor. Analysis of Biological Development. 2nd ed: McGraw-Hill Science/Engineering/Math; 2000.
30. Larsen WJ, Sherman LS, Potter SS, Scott WJ. Human Embryology. 3rd ed: Churchill Livingstone; 2001.
31. Levran D, Nahum H, Farhi J, Weissman A. Poor outcome with round spermatid injection in azoospermic patients with maturation arrest. Fertil Steril 2000;74(3):443-49.
32. Lie RT, Lyngstadaas A, Orstavik KH, Bakketeig LS, Jacobsen G, Tanbo T. 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.
33. Moskovtsev SI, Willis J, White J, Mullen JB. Leukocytospermia: Relationship to sperm deoxyribonucleic acid integrity in patients evaluated for male factor infertility. Fertil Steril 2007;88(3):737-40.
34. Munoz-Garay C, De la Vega-Beltran JL, Delgado R, Labarca P, Felix R, Darszon A. Inwardly rectifying K^+ channels in spermatogenic cells: Functional expression and implication in sperm capacitation. Dev Biol 2001;234(1):261-74.
35. Nagy ZP, Joris H, Verheyen G, Tournaye H, Devroey P, Van Steirteghem AC. Correlation between motility of testicular spermatozoa, testicular histology and the outcome of intracytoplasmic sperm injection. Hum Reprod 1998;13(4):890-95.
36. Palermo GD, Neri QV, Takeuchi T, Hong SJ, Rosenwaks Z. Intracytoplasmic sperm injection: Technical aspects. In: Gardner DK, Weissman A, Howles CM, Shoham Z, editors. Textbook of Assisted Reproductive Technologies: Laboratory and Clinical Perspectives. Third ed: Taylor and Francis, Inc.; 2008:171-80.
37. Primakoff P, Myles DG. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. Science. 2002;296(5576):2183-85.
38. Publicover SJ, Barratt CL. Voltage-operated Ca^{2+} channels and the acrosome reaction: which channels are present and what do they do? Hum Reprod 1999;14(4):873-79.
39. Purohit SB, Laloraya M, Kumar GP. Role of ions and ion channels in capacitation and acrosome reaction of spermatozoa. Asian J Androl 1999;1(3):95-107.
40. Reefhuis J, Honein MA, Schieve LA, Correa A, Hobbs CA, Rasmussen SA. Assisted reproductive technology and major structural birth defects in the United States. Hum Reprod. 2009;24(2):360-66.
41. Scott L. Analysis of Fertilization. In: Gardner DK, Weissman A, Howles CM, Shoham Z, editors. Textbook of Assisted Reproductive Technologies: Laboratory and Clinical Perspectives. Third ed: Taylor and Francis, Inc.; 2008:207-17.
42. Spandorfer SD, Avrech OM, Colombero LT, Palermo GD, Rosenwaks Z. Effect of parental age on fertilization and pregnancy characteristics in couples treated by intracytoplasmic sperm injection. Hum Reprod. 1998;13(2):334-38.
43. Spandorfer SD, Davis OK, Barmat LI, Chung PH, Rosenwaks Z. Relationship between maternal age and aneuploidy in in vitro fertilization pregnancy loss. Fertil Steril 2004;81(5):1265-69.
44. Strohsnitter WC, Noller KL, Hoover RN, et al. Cancer risk in men exposed in utero to diethylstilbestrol. J Natl Cancer Inst 2001;93(7):545-51.
45. Swain JE, Pool TB. ART failure: Oocyte contributions to unsuccessful fertilization. Hum Reprod Update 2008; 14(5):431-46.
46. Tesarik J, Sousa M. Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dislocation. Fertil Steril 1995;64(4):770-76.
47. Varghese AC, du Plessis SS, Agarwal A. Male gamete survival at stake: Causes and solutions. Reprod Biomed Online 2008;17(6):866-80.
48. Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod. 1998;59(1):1-6.
49. Zamir N, Riven-Kreitman R, Manor M, et al. Atrial natriuretic peptide attracts human spermatozoa in vitro. Biochem Biophys Res Commun 1993;197(1):116-22.

SECTION 2

Clinical Andrology

CHAPTER 4

Pathophysiology of Male Infertility

Kamini A Rao, Baiju PA

INTRODUCTION

Major functions of human testes are steroidogenesis and spermatogenesis. Both these events are under the endocrine and paracrine control. Endocrine control is mediated through gonadotropins which are in turn controlled by pulsatile GnRH secretion from hypothalamus which reaches pituitary gland through hypophyseo-pituitary portal system. Paracrine control involves the mediation of many growth factors. Sertoli cells are the supporting cells in the seminiferous tubules, which has a major role in the spermatogenesis. They are also known as Sustentacular cells. Leydig cells are seen in the interstitium of testes produce testosterone and they also have an important role in the control of spermatogenesis. Tight junctions between the Sertoli cells also play a major regulatory role in spermatogenesis.

In this chapter we will concentrate on the physiological and pathological changes occurring in testes which contribute to infertility.

SPERMATOGENESIS

Spermatogenesis was first described by Lerblond and Clermont in 1952 in rats. Spermatogenesis can be divided into three stages which includes (1) Clonal Expansion (2) Maturation (3) Differentiation. Clonal expansion and maturation stages together known as Spermatocytogenesis and Differentiation stage is also known as Spermiogenesis.

Spermatogenesis (Figure 4.1) represents the process by which precursors, spermatogonia undergo a complex series of divisions to give rise to spermatozoa.[1, 2] This process takes place within the seminiferous epithelium which is a complex structure composed of germ cells and radially oriented supporting cells called Sertoli cells. The latter cells extend from the basement membrane of the seminiferous tubules to reach the lumen (Figure 4.2).

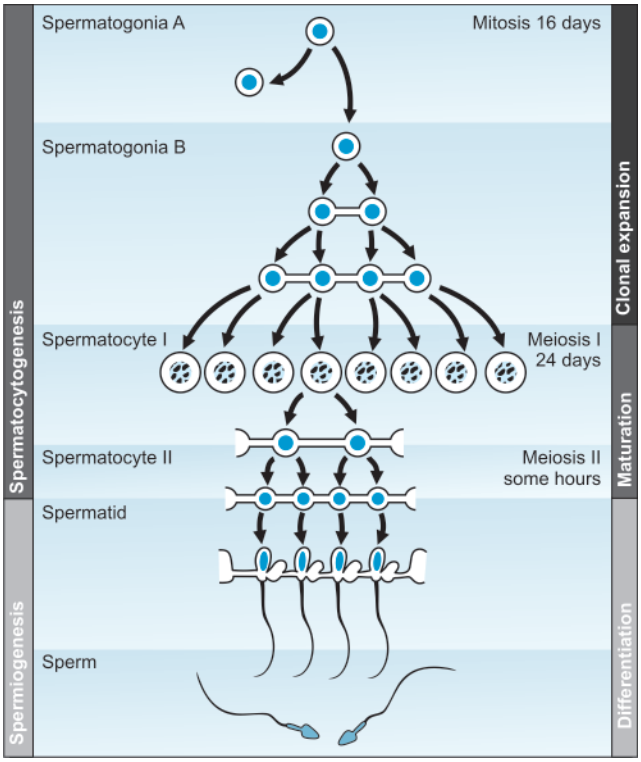


Figure 4.1: Spermatogenesis

In general two main classes of spermatogonia can be identified in humans: Type A exhibiting fine pale staining nuclear chromatin and Type B with coarse chromatin

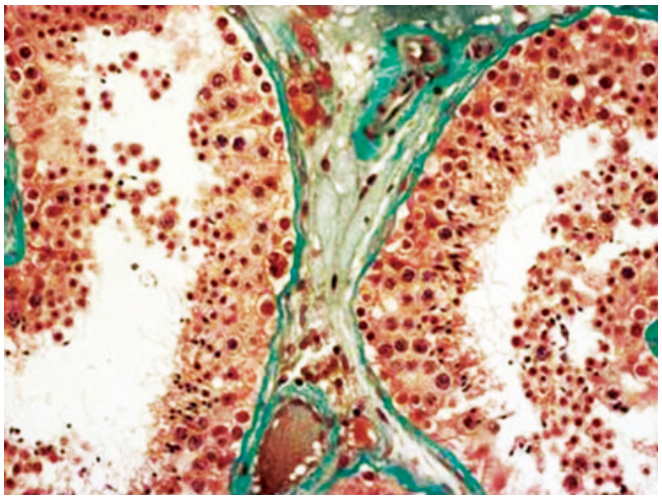


Figure 4.2: Seminiferous tubules

collections found close to the nuclear membrane.[3] The Type B spermatogonia are generally agreed to represent the cells which differentiate and enter into the process of meiosis where they are called primary spermatocytes. Type A spermatogonia can be further divided into A dark (Ad) and A pale (Ap).[4] Some investigators have proposed that the Ad spermatogonia represent the reserve or non-proliferative spermatogonial population which can give rise to Ap[5-7] whereas others have suggested that the Ap spermatogonia are the true stem cell of the testis.[8]

Meiosis

Meiosis commences when Type B spermatogonia lose their contact with the basement membrane to form preleptotene primary spermatocytes. The preleptotene primary spermatocytes engage in DNA synthesis and condensation of individual chromosomes providing the appearance of thin filaments in the nucleus which identify the leptotene stage.[9] At this stage, each chromosome consists of a pair of chromatids (Figure 4.3). As the cells move into the zygotene stage, there is further thickening of these chromatids and the pairing of homologous chromosomes. The further enlargement of the nucleus and condensation of the pairs of homologous chromosomes termed bivalents, provides the nuclear characteristics of the pachytene stage primary spermatocyte. During this stage, there is exchange of genetic material between homologous chromosomes derived from maternal and paternal sources. The sites of exchange of genetic material are marked by the appearance of chiasmata and these become visible when

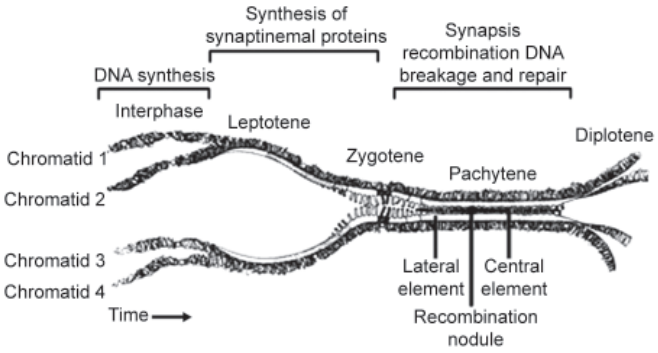


Figure 4.3: Meiotic events in spermatogenesis

the homologous chromosomes separate slightly during diplotene. The exchange of genetic material involves DNA strand breakage and repair.[10]

The diplotene stage is recognized by partial separation of the homologous pairs of chromosomes that still remain joined at their chiasmata and each is still composed of a pair of chromatids. With dissolution of the nuclear membrane, the chromosomes align on a spindle and each member of the homologous pair moves to opposite poles of the spindle during anaphase. The resultant daughter cells are called secondary spermatocytes and contain the haploid number of chromosomes but, since each chromosome is composed of a pair of chromatids, the DNA content is still diploid. After a short interphase, which in the human represents approximately six hours, the secondary spermatocytes commence a second meiotic division during which the chromatids of each chromosome move to opposite poles of the spindle forming daughter cells that are known as round spermatids.[11,12] Meiotic maturation in the human takes about 24 days to proceed from the preleptotene stage to the formation of round spermatids (Table 4.1).

Spermiogenesis

The transformation of a round spermatid into a spermatozoon represents a complex sequence of events that constitute the process of spermatogenesis. No cell division occurs but a conventional round cell becomes converted into a spermatozoon with the capacity for motility. The basic steps in this process are consistent between all species and consists (a) the formation of the acrosome (b) nuclear changes (c) the development of the flagellum or sperm tail (d) the reorganization of the cytoplasm and cell organelles and (e) the process of release from the Sertoli cell termed spermiation.[13, 14]

Table 4.1: The temporal course of spermatogenesis

Mitosis	24 Days	Up to Primary Spermatocyte
First Meiosis Secondary	16 Days	Primary Spermatocyte to Spermatocyte
Second Meiosis	Few hours	For engendering Spermatids
Spermiogenesis	24 Days	Up to Mature Spermatozoa
Total	64 Days	

CONTROL OF SPERMATOGENESIS

Spermatogenesis is a complex process and control of spermatogenesis involves many factors. Major control can be divided and discussed under 3 major headings.

1. Anatomical Factors
2. Classic Endocrine Factors
3. Intrinsic Factors.

Anatomical Factors

Anatomical factors which control spermatogenesis are (A) Temperature and (B) Pressure inside the testis. The scrotal temperature is 2-3 degree less than the core body temperature.

Endocrine Control of Spermatogenesis

Classic endocrine control of spermatogenesis is given in the following diagram (Figure 4.4).

Testosterone has got a negative feedback on hypothalamus and controls GnRH secretion, where as estradiol which is produced as a result of aromatase action, has got a negative feedback on hypothalamus and pituitary gland.

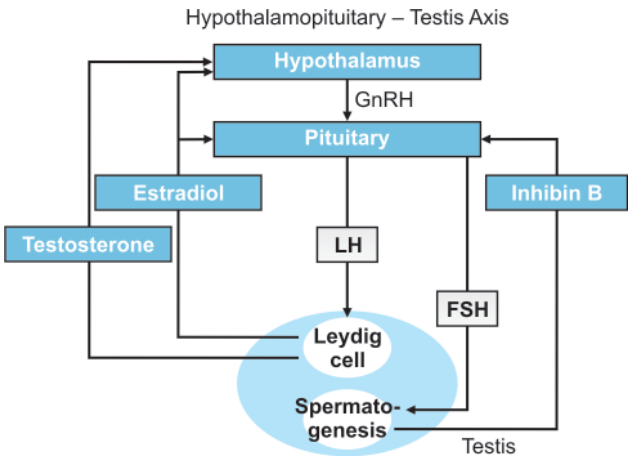


Figure 4.4: Endocrine control of spermatogenesis

INTRINSIC CONTROL OF SPERMATOGENESIS

The spermatogenic process is controlled by both classic endocrine mechanisms as well as by intrinsic mechanisms that are mediated by growth factors, cytokines and other molecules.[15] Pivotal in our understanding of intrinsic mechanisms is the central role played by the Sertoli cell. As discussed earlier, the Sertoli cell, through the formation of the blood-testis barrier divides the seminiferous epithelium into a basal and adluminal compartment and limits intercellular transport to the centrally placed germ cells.[16] These cells therefore control the environment in which all germ cells other than spermatogonia develop.

Factors involved in the intrinsic control of spermatogenensis are the following:

1. Lactate
2. Transferrin
3. Ceruloplasmin
4. Vitamin A
5. Thyroid hormone
6. Stem cell factor

Sertoli cell play an important role in the intrinsic control of spermatogenesis by modulating the entry of substances into the adluminal compartment of seminiferous tubules negotiating the blood testes barrier.

1. Lactate

Several studies have been established that lactate rather than glucose is the preferred substrate for glycolysis in primary spermatocytes. Lactate is generated from glucose by Sertoli cells under the influence of FSH. Production of lactate will lead to changes in pH resulting in the alterations of processing of the stem cell factor from a soluble cell bound form and thereby influencing stem cell factor's action on spermatogonial stimulation.[17]

2. Transferrin

There are a range of proteins produced from Sertoli cells mediated through FSH, but they cannot gain access to the seminiferous tubular epithelium because of the presence of blood testes barrier. Testicular transferrin is a secretory product of Sertoli cell. It helps in various stages of spermatogenesis by supplying iron to the spermatocytes. Transferrin supplies iron to the primary spermatocytes through receptor mediated endocytosis. Iron is an essential element to maintain respiration and cytochorome function.[18]

3. Ceruloplasmin

Ceruloplasmin is a copper binding protien. It is involved in the spermatogenesis by supplying copper which is required for coenzyme function for a number of proteins. Ceruloplasmin delivers copper to germ cells which is also controlled by the Sertoli cell through FSH.[19]

4. Vitamin A

Vitamin A has a dual role in the spermatogenesis. It has a direct and an indirect action. Indirect action is through acting on Sertoli cell and increasing the secretory products which helps in spermatogenesis. Direct action is by attaching to retinoid receptors on Sertoli cells and helping in differentiation of germ cells.[20, 21]

5. Thyroid Hormone

Along with FSH action, thyroid hormone, thyroxine can also act directly on Sertoli cell and exert its proliferative effect and in this way they can control spermatogenesis.[22-24]

6. Stem Cell Factor

Recent studies suggest that the production of a membrane bound form of stem cell factor by alternative splicing may be essential for spermatogonial multiplication.[25] The production of the membrane-bound form is stimulated by a decrease in pH facilitated by the generation of lactate by Sertoli cells, an essential element in facilitating the survival of primary spermatocytes whose preferred substrate for glycolysis is lactate.[26] This is an example of the close interaction between Sertoli cells and germ cells both in the production of growth factors and the interplay of their metabolic pathways.

PATHOPHYSIOLOGY OF MALE INFERTILITY

Type of Infertility	*Frequency (%)*
Untreatable sterility	12%
Primary seminiferous tubule failure	12%
Treatable conditions	18%
Sperm autoimmunity	7%
Obstructive azoospermia	10%
Gonadotropin deficiency	0.5%
Disorders of sexual function	0.5%
Reversible toxin effects	0.02%
Untreatable subfertility	70%
Oligospermia	35%
Asthenospermia and teratozoospermia	30%
Normospermia with functional defects	5%

As we all know, the testis has an endocrine as well as an exocrine function. Endocrine testicular failure results in testosterone deficiency. In primary endocrine testicular failure, a decline in testosterone secretion–hypoandrogenism, is caused by a deficiency or absence of Leydig cell function. Clinically important problems to be discussed under the heading of primary endocrine testicular failure include anorchia, Leydig cell hypoplasia, numerical - structural chromosome abnormalities and testicular dysgenesis. Secondary endocrine testicular failure is caused by absent or insufficient bioactivity of GnRH or LH. Primary exocrine testicular failure is caused by Cryptorchidism, Hypospadiasis and Testicular tumors, especially seminoma of testes, are also discussed in this chapter.

ANORCHIA

Anorchia is the absence of testicular tissue in a phenotipically and genetically male patient. It can be unilateral or bilateral. Anorchia can be congenital or acquired (rather most commonly it is iatrogenic). Unilateral congenital anorchia is common compared to the bilateral which is extremely rare.

CONGENITAL ANORCHIA

Development of external genitalia is solely dependent on the presence of Anti-Müllerian Hormone (AMH) and androgen–Dihydrotestosterone. In congenital bilateral anorchia testes usually disappear after its initial appearance. For the normal development of Wolffian duct structures, at least one testis must be present up to the 16th week of gestation, but later its disappearance will not affect the development of Wolffian structures. (Which is known as "the vanishing testis syndrome"). Intrauterine infarction of a maldescended testis or Intrauterine testicular torsion appears to be the major leading contributory factors to anorchia.[26, 27]

Usually patients with bilateral anorchia will have elevated FSH and LH and they will rise further to a very high level after the onset of puberty. But the Testosterone hormone will be in very low level which is equal to the castrated level. Differential diagnosis of complete anorchia will include cryptorchidism and testicular atrophy. Assay of serum AMH and hCG test will give the definite diagnosis. After hCG administration, testosterone levels remain unchanged in patients with bilateral anorchia even after a 7-day period of post

stimulation, while a rise can be detected in patients with cryptorchidism because of the presence of intra abdominal testes. Serum AMH is undetectable in cases of anorchia. It has a higher sensitivity compared to hCG test, but equal specificity for differentiation of bilateral anorchia from bilateral cryptorchidism. Endocrine tests are not really useful for differential diagnosis of unilateral anorchia. In these cases CT or MRI and finally exploratory surgery or laparoscopy will give a definite final opinion.[28-30]

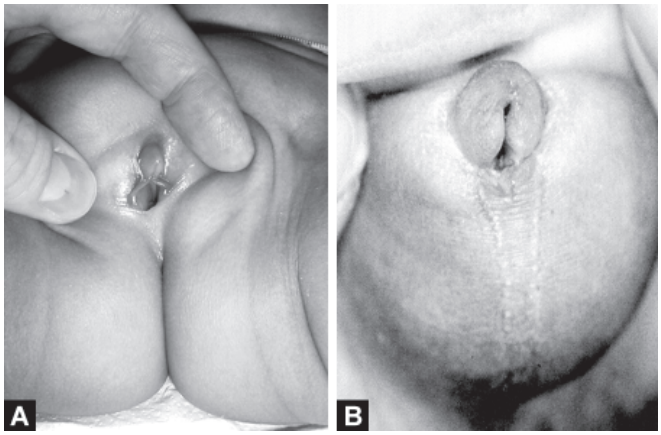
ACQUIRED ANORCHIA

It can be unilateral or bilateral. Most common cause for bilateral acquired anorchia is removal of both testes in androgen dependent carcinoma prostate. Other causes of anorchia includes unintended removal or devascularization during herniotomy or orchidopexy, testicular infarction, severe trauma and very rarely self-mutilation. If only one testis is lost then fertility and testosterone production will normally be maintained by the remaining testis and no specific therapy is required. Acquired anorchia before puberty leads to the characteristic phenotype of male eunuchoidism and but if it happens after puberty, characteristic features are of testosterone deficiency.[31]

LEYDIG CELL HYPOPLASIA

It is a rare condition and it is inherited through autosomal recessive pattern. Incidence of Ledig cell hypolasia is 1 in 1,000,000. LH receptor inactivating mutations will cause defective development of Leydig cells. This mutation causes failure of necessary stimulation of intracellular pathways. Men with Leydig cell hypoplasia usually present with very low serum testosterone and high LH levels.

There are two types of Leydig cell hypoplasia (Figure 4.5) described in the literature. Type I—Severe form, resulting in female external genitalia with blind ending vagina, primary amenorrhea and absence of secondary sex differentiation at puberty. It is caused by inactivating mutations in the LH receptor that completely prevent LH and hCG signal transduction and thus complete blockade of testosterone production. Leydig cell hypoplasia Type II— Mild form and is characterized by milder signs of androgen deficiency with a predominantly male phenotype but features of hypogonadism with micropenis and usually associated hypospadia. This milder form is originated from mutations of the LH



Figures 4.5A and B: Complete and incomplete Leydig cell hypoplasia

receptor, which is only partially inactivate signal transduction and retains some responsiveness to LH. Histology of testes shows seminiferous tubules, whereas Leydig cells are not present or if at all present appear as immature forms. Epididymides and deferent ducts are usually seen, whereas Müllerian structures—uterus, tubes or upper vagina are not developed.[32-34]

SPERMATOGENIC FAILURE

Germ Cell Aplasia (Sertoli-Cell-Only Syndrome)

Germ cell aplasia or Sertoli-cell-only (SCO) syndrome is a histopathologic diagnosis which was first described by Del Castello in 1947. It can be complete or focal variety and also congenital and acquired. In complete germ cell aplasia (Figure 4.6) the tubules are reduced in diameter, and contain only Sertoli cells but no other cells involved in spermatogenesis. Germ cell aplasia can also be focal with

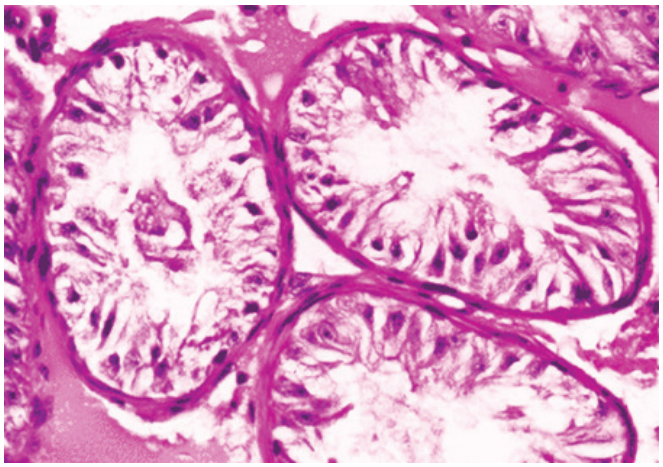


Figure 4.6: Germ cell aplasia

a variable percentage of tubules containing germ cells, but in these tubules spermatogenesis is often limited in both quantity and quality, but such cases are usually referred to as hypospermatogenesis. Germ cell aplasia or SCO syndrome is one of the common causes of non-obstructive azoospermia.[35, 36]

In congenital germ cell aplasia the primordial germ cells fail to migrate from the yolk sac into the gonads or unable to survive in seminiferous tubular epithelium. Chromosomal abnormalities, especially microdeletions of the Y chromosome, are important genetic causes for complete congenital germ cell aplasia. Anti cancer therapy with radiotherapy or chemotherapy may cause complete acquired loss of germ cells. Other acquired causes include viral infections of the testes such as mumps orchitis. Germ cell aplasia can occur in maldescended or undescended testes also.[37]

Diagnosis of germ cell aplasia can be made only by testicular biopsy. However, the testicular biopsy may not be representative always and in few patients, testicular sperm have been retrieved by TESE who are previously diagnosed to have "complete germ cell aplasia" by testicular histology. It has been documented in literature that chances of successful sperm retrieval in cases of complete germ cell aplasia are about 25%. Therefore, multiple testicular biopsies of both testes must be screened carefully before making a diagnosis of complete germ cell aplasia.[38, 39]

SPERMATOGENIC ARREST

Spermatogenic arrest is also a specific diagnosis made by histopathological description of the interruption of normal germ cell maturation at the level of a specific cell type. It includes spermatogonial arrest, spermatocyte arrest and spermatid arrest. A definite diagnosis can only be made by multiple testicular biopsies. Testicular volume, FSH and inhibin B may be within their normal ranges, but can also be elevated or depressed (Figure 4.7).

The causes of arrest can be congenital or acquired. Congenital causes are basically of gentic origin. These genetic etiologies include trisomy, balanced-autosomal anomalies like translocations, inversions or deletions in the long arm ofY chromosome (Yq11). All the genetic causes are not yet completely identified.[40] The suspected role of several gene mutations and polymorphisms has been extensively investigated but no clear-cut genetic factor could be identified so far. So much so, till date no gene mutation or polymorphism analysis has shown adequate reliable sensitivity and specificity as a diagnostic tool in the workup of male infertility.[41-43]

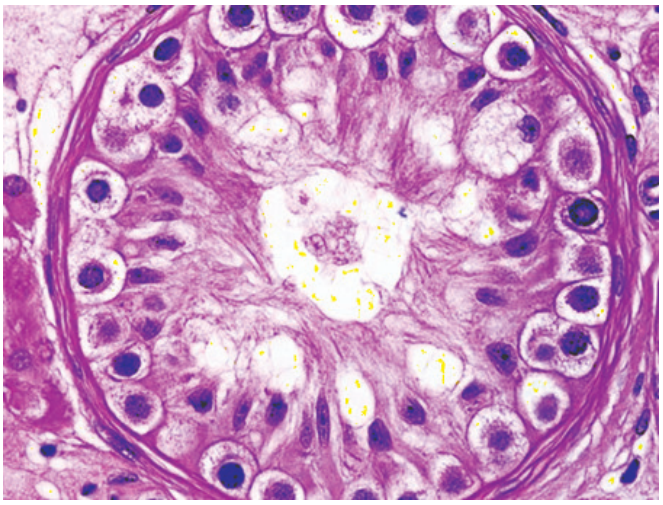


Figure 4.7: Spermatogenic arrest

Secondary causes for spermatogenetic arrest are toxic substances—radiotherapy, chemotherapy, antibiotics, elevated temperature or general illnesses (liver or kidney failure, blood dyscrasias like sickle cell anemia).[44,45]

HYPOSPERMATOGENESIS (FIGURE 4.8)

This is another histological diagnosis of primary exocrine testicular failure in which all germ cell types, including mature spermatids, is present in some or all tubules, but reduced in number. According to the intensity of the

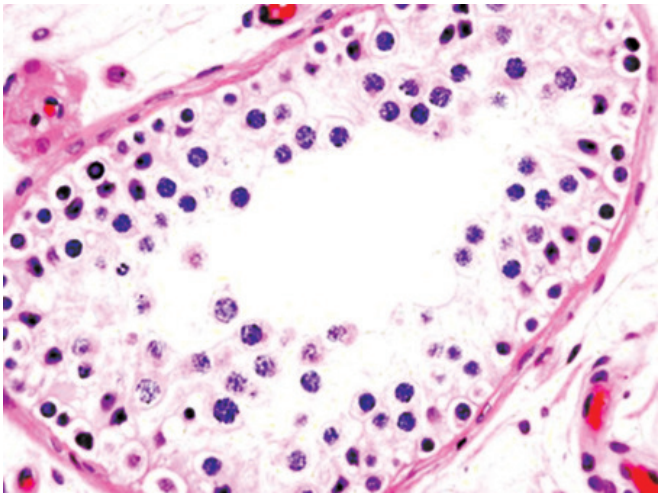


Figure 4.8: Hypospermatogenesis

problem it can be divided into Mild, Moderate, and Severe forms. Some cases complete germ cell aplasia in some seminiferous tubules but with complete spermatogenesis in the adjacent tubules (often known as 'focal' germinal cell aplasia) while other patients will have the appearance of an increased number of germ cell precursor compared to the number of mature spermatids in the seminiferous epithelium. These cases are known as incomplete or focal germinal cell aplasia.

Etiologies of all these cases are similar to the causes of germinal cell aplasia. From a practical clinical point, the differentiation is important as patients with hypospermatogenesis may have azoospermia or varying degrees of oligoasthenoteratozoospermia and sperm can be retrieved from testis by TESE.[46]

Commonly in patients with hypospermatogenesis testicular volume is low. FSH level is elevated in most cases, but need not be in all patients. Usually high serum FSH levels correlate positively with the proportion of tubules with germ cell aplasia.[47] But many studies have demonstrated the inhibin B value is a more sensitive and specific endocrine marker of hypospermatogenesis than FSH.[48,49] However, even the combined measurement of inhibin B and FSH provides no strong association with the presence or absence of sperm in TESE.[50, 51]

In cases of severe hypospermatogenesis, pregnancies can be achieved with TESE and ICSI. Role of FSH therapy in these cases are controversial. However, randomized studies performed till date has demonstrated marginal increase in total sperm count in the ejaculate but without any significant effect on fertility.[52]

NUMERICAL CHROMOSOME ABNORMALITIES

Klinefelter's Syndrome (Figure 4.9)

This syndrome was first described by Harry Klinefelter in 1942. But later, only in 1959 the chromosomal basis of the disorder was first described. Clinical features include bilateral small testes, azoospermia, gynecomastia and a high serum FSH. Diagnosis of Klinefelter syndrome is by the demonstration of the 47, XXY karyotype or one of its other mosaic variants.[53]

The prevalence of Klinefelter's syndrome appears to be approximately 1 in 660 males, and new data suggest an increasing incidence over the last few years. It is the most common form of primary testicular failure affecting spermatogenesis as well as hormone production affecting both steroidogenic axis and spermatogenic axis of testicular function. It is found in about 12% of men presenting with

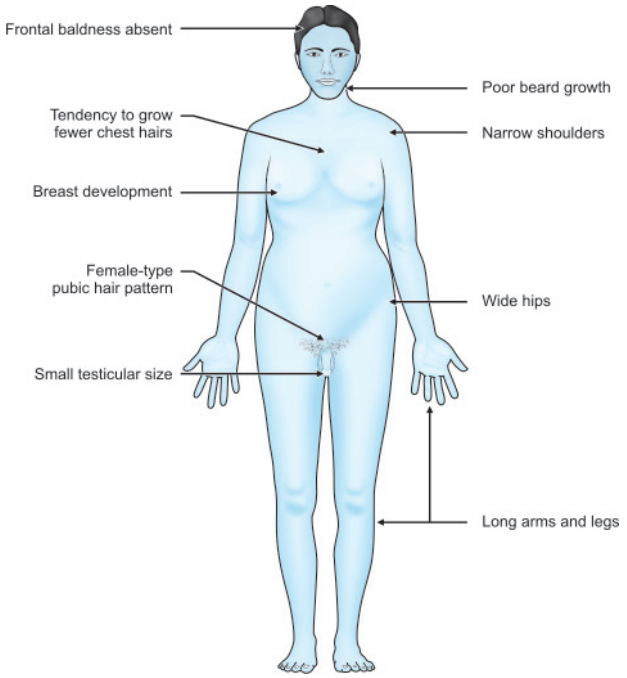


Figure 4.9: Clinical features of Klinefelter's syndrome

azoospermia. But interestingly it is noticeable that 50% of the cases remain undiagnosed and untreated throughout life.[54, 55]

A non-mosaic 47, XXY karyotype is the most common chromosomal pattern and seen in 80-90% of Klinefelter syndrome patients. Mosaic pattern is seen in approximately 5-10% of patients. Among mosaic pattern, 47, XXY/46, XY mosaicism is most commonly seen abnormality. The 48, XXXY, 48, XXYY and 49, XXXXY are seen very rarely. Structurally abnormal extra X chromosomes are seen in less than 1% of patients. Molecular genetics methods can also be used to quantify the number of X chromosomes in patient and is done by quantitative PCR analysis of the androgen receptor gene located on the X chromosome. This analysis supposed to be more accurate in quantification of X chromosoes.[56]

The extra X chromosome in non-mosaic 47, XXY patient is derived during meiotic error. Chance of that meiotic error to occur is equally shared by maternal and paternal chromosomes. Most often this occur by meiosis without X/Y or X/X recombination. Advanced maternal age is a known risk factor. It is not very clear whether the 47, XXY karyotype is over-represented among spontaneous early abortions. However, actually compared to other aneuploidies, Klinefelter's syndrome appears to be only a minor risk factor and most often pregnancies result in a live-birth without any problem.[57]

Diagnosis of Klinefelter's syndrome is inconspicuous until puberty. Usually height gain growth velocity is increased in the immediate pre-pubertal period. Men with Klinefelter's syndrome are usually tall and to have relatively long legs and decreased upper segment to lower segment ratio (Figure 4.10).

Commonly early period of puberty proceed normally and uneventfully. The syndrome is typically characterized by the small testes with firm consistency and small volume with a range of 1-4 ml. Infertility due to azoospermia in these cases. Testicular histopathology of Klinefelter's syndrome displays different patterns Figure 4.11. Most common features are germ cell aplasia, total tubular atrophy or hyalinizing fibrosis and relative hyperplasia of Leydig cells are found. However, foci of spermatogenesis up to the stage of mature testicular sperm can be visualized in some cases of Klinefelter patients.[58, 59]

In early stages, serum LH and FSH increase while serum levels of testosterone remain stationary or just below the normal range of an adult normal male. Compared to LH, FSH levels are exceedingly high; serum levels of inhibin B are usually undetectable. But serum estradiol levels are high normal or may exceed the normal range. After the age of 25 years, approximately 80% of patients will have decreased serum testosterone levels and symptoms of androgen deficiency.[60, 61]

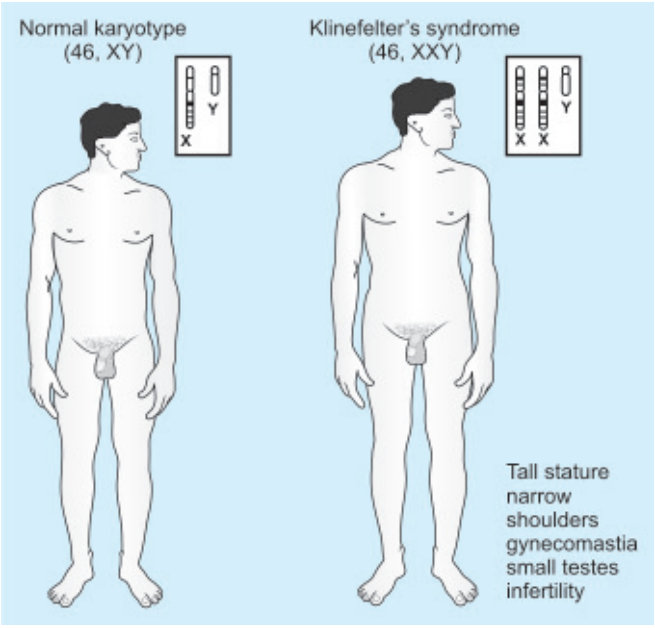


Figure 4.10: Comparison of phenotype in a normal male to Klinefelter's syndrome

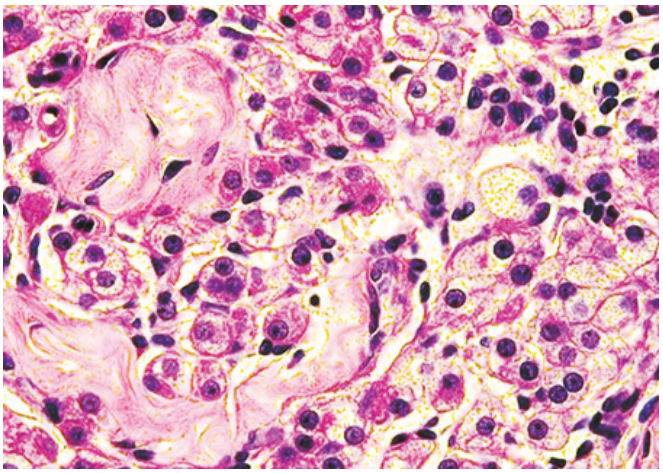


Figure 4.11: Testicular histology in Klinefelter's syndrome

During puberty bilateral gynecomastia of varying degrees develops in approximately 50% of the patients. The risk of developing cancer breast is increased relatively compared to normal men but remains very rare. But there is highly increased chances for the development of mediastinal malignant germ cell tumor. It usually occur at the age between 14 and 29 years.[62, 63]

The intelligence of Klinefelter patients is very variable. Usually the intelligence quotient is 11 points less compared to a normal male of comparable age. The deficits are observed commonly in verbal and cognitive abilities. Usually mosaic Klinefelter patient exhibit normal or low normal IQ compared to the classical ones.[64,65]

The pattern of inactivation of one copy of the androgen receptor gene reflects the variability of the clinical features in patients with Klinefelter's syndrome and it is related to degree of androgenisation.[66]

Very rarely sperm can be seen in the ejaculate and occasionally in TESE samples. The diploidy of sperm as well as disomy for sex chromosomes and autosomal chromosomes seems to be increased in patients with Klinefelter's syndrome. However, the majority of sperm appear to be normal if retrieved or seen in ejaculate. It is estimated that approximately 20-50 percent of patients with Klinefelter's syndrome it is possible to retrieve sperm by TESE. Pregnancies have been achieved with testicular sperm after doing ICSI. Commonly, the birth of normal children conceived by assisted reproductive techniques seems to be the rule. The possible explanation is that the few sperm which can be found in about 50% of patients with Klinefelter's syndrome are usually

derived from the clonal expansion of spermatogonia with normal karyotype.[67-75]

XX-MALE SYNDROME

The XX-male syndrome is featured by the presence of male external genitalia, testicular differentiation of the gonads and a 46, XX karyotype in a routine conventional cytogenetic analysis. This problem shows a prevalence of 1:9,000 to 1:20,000.

Molecular methods have been demonstrated that about 75% of XX-males have Y chromosomal material translocated on to the tip of one X chromosome. Translocation of a DNA-segment containing the testis-determining gene (SRY=Sex Determining Region Y) from the Y to the X chromosome usually happens during paternal meiosis. The presence of the gene causes the initial indifferent gonad to development into a male differentiated gonad.[76, 77]

Majority of SRY-positive individuals are very much similar to patients with Klinefelter's syndrome. But characteristic differentiating factor is that 46, XX males are significantly shorter than Klinefelter patients or normal healthy men, resembling their female counterparts in height and weight. The incidence of maldescended testes is much higher than that in Klinefelter patients and normal healthy men. The testes are very small (1-4 ml) and firm, and endocrine profile shows decreased serum testosterone and elevated estrogen and gonadotropin levels. Approximately 50% patient develops gynecomastia. But their intelligence is normal, but, this is questionnable. Semen analysis reveals azoospermia. The testicular histology of XX males with positive SRY factor shows atrophy and hyalinization of the seminiferous tubules and they are devoid of germ cells.[78]

But in cases of SRY-negative XX-males the mechanism underlying the sex reversal remains unclear. Almost 25% of XX Males are negative for SRY gene. These patients show less virilization than SRY-positive individuals and associated mlformations include undescended testes, bifid scrotum and hypospadiasis.[79]

XYY-SYNDROME

Most 47, XYY males have normal health compared to normal XY males. The prevalence among unselected newborns appears to be 1:1000. Diagnosis is done by demonstration of two Y chromosomes in an otherwise normal karyotype. This chromosomal aneuploidy is caused by non-dysjunction in paternal meiosis. Most of the time it is detected incidentally when karyotyping has done for some other pupose. Endocrine profile of these patients reflects serum levels of testosterone and gonadotropin levels are in normal male range. So much so, the volume of testes is also comparable to those of normal healthy men. Usually they have delayed puberty approximately by 6 months. They can also have abnormal seminal parameter ranging from severe oligospermia to Azoospermia. But most men with 47, XYY-syndrome have normal fertility. Onset of puberty seems to be delayed by 6 months. Usually they are tall and adult height is 7 cm in excess of the mean male population. The IQ is lies within the normal range, but men score an average of ten points less than age-matched peers. Behavioral problems are more common in 47, XYY males; however, a history of violent behavior is exceptional. Most 47, XYY-men do not need any specific therapy. Men who achieve fatherhood can expect chromosomally normal offspring probably with the same likelihood as normal men. Nevertheless, to be on safe side, it is better to offer prenatal diagnostic techniques to these children.[80,81]

Numerical Chromosome Abnormalities Limited to Spermatozoa

These anomalies cannot be picked up by the somatic cell karyotyping because they are strictly confined to the sex chromosomes. These anomalies (XY, XX, and YY disomy, and diploidy) are inversely propotional to the sperm concentration of these individuals. So usually they present with severe oligozoospermia.[82-84]

STRUCTURAL CHROMOSOME ABNORMALITIES

Structural chromosome abnormalities comprise pathological alterations of chromosome structure that are detectable through light-microscopic examination of banded metaphase preparations in routine analysis. It can be seen in both autosomal chromosomes and sex chromosomes. Classically, structural rearrangements such as Robertsonian translocations are also considered as structural abnormalities.

Structural anomalies of the autosomes should be differentiated from anomalies of the sex chromosomes. Among structural chromosomal anomalies, Reciprocal and Robertsonian translocations, inversions, marker

chromosomes X and Y isochromosomes and Y chromosomal deletions are of practical importance in male infertility.

The important factor in the assessment of structural chromosomal anomalies as far as the clinical practice is concerned, is the differentiation between balanced and unbalanced chromosomal translocations. The former are characterized by a deviation from normal chromosome structure but without a net loss or gain of genetic material. If no important gene is disrupted at the breakpoints, balanced structural aberrations exert no negative effect on general health. However, the balanced abnormalities can adversely affect fertility and could give rise to unbalanced karyotypes among the children which can cause early abortions and recurrent pregnancy losses.[85-88]

In cases of unbalanced structural chromosomal abnormalities, there are two possibilities—genetic material is either missing or there is a net excess of material inside the cell. Majority of all unbalanced karyotypes are usually associated with severe disturbances of general health, if they are compatible with survival. Exceptions are Y chromosome deletions that may limit reproductive functions selectively without affecting general health, and therefore they are of importance in reproductive medicine.

The majority of males carrying structural anomalies are fertile and don't require specific therapy. But men with impaired spermatogenesis show an increased prevalence of structural chromosomal abnormalities. Infertile patients with structural chromosomal aberrations may conceive naturally or more severe cases may require treatment modalities such as intracytoplasmic sperm injection. However, success rates of these interventions may be lower than in couples with normal karyotypes. It should also be kept in mind that unbalanced karyotypes of the embryo can result from balanced parental chromosomal aberrations. For any carrier of a structural chromosome abnormality, if they are planning for fertility treatment, genetic counseling is recommended very strongly before any kind of infertility treatment. It should be mentioned that karyotyping of men with idiopathic infertility and decreased sperm concentration is usually recommended prior to IVF-ICSI treatment. The risks associated with these chromosomal aberrations, options of pre-implantation genetic diagnosis, if possible and definitely prenatal diagnostic tests should be offered and for certain aberrations, the possibility that other family members are also affected should be discussed with the patient.[89-92]

Structural Abnormalities of the Autosomal Chromosomes

Balanced autosomal anomalies adversely affect spermatogenesis by interfering with the meiotic pairing of the chromosomes. These abnormalities commonly do not demonstrate a typical clinical phenotype. The extent of disturbed fertility cannot be foreseen in individual cases. The same balanced autosomal aberration can have a severe effect on spermatogenesis in one patient and none at all in another patient showing its variable degree of expression. Even siblings with similar pathological karyotype can have widely differing sperm concentrations. So far no clinical or laboratory parameter in an infertile male is known which reliably indicates the presence of an autosomal structural anomaly. Therefore, in cases of unclear azoospermia or severe oligozoospermia karyotyping is generally advised.[93]

Translocations and inversions usually represent cases of familial chromosomal aberrations. In these patients a pedegree analysis should be encouraged, as the presence of a translocation or inversion is often associated with a higher rate of abortions, recurrent pregnancy losses and, in some cases, the risk for the birth of a severely malformed baby.[94]

Structural Abnormalities of Sex Chromosomes

An intact Y chromosome is essential for the normal function of male reproductive system. The male-specific region of the Y chromosome causes differentiation of the sex. The SRY gene is located on the short arm of the Y chromosome and it guides differentiation of the embryonic gonad in to the testicular pathway and hence development of internal and external male genitalia. The long arm of the Y chromosome contains areas responsible for the normal spermatogenesis.[95]

While speaking about deletions of the Y chromosome, those of the short and the long arm must be understood. Short arm deletion of the Y chromosome contains the sex determining SRY gene result in sex change or reversal (Figure 4.12). Clinically, affected men appear as phenotypically female individuals with somatic stigma of Turner's syndrome. If the deletion affects the long arm, there will not be any change in the phenotype. With the loss of the heterochromatic part of the Y chromosome's long arm (Yq12) general and reproductive health are unaffected. Deletions of the euchromatic part of the long arm of Y chromosome (Yq11) may affect spermatogenesis, because Yq11 harbors locus which is essential for normal spermatogenesis.[95,96]

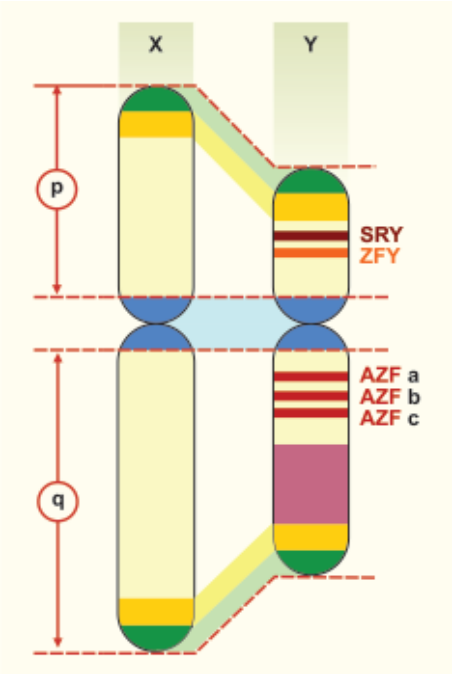


Figure 4.12: Structural comparison of X and Y chromosomes

Apart from deletions, a series of structural anomalies of the Y chromosome also exist. Pericentric inversions are usually without any consequences. An isodicentric Y chromosome is a more complex aberration nearly always occurring as a mosaic with a 45, X-cell line. The phenotype can be male, female or intersex. Patients with a male phenotype are usually infertile. These patients have an increased chance of developing testicular tumors.[96]

Reciprocal translocations between the Y chromosome and one of the autosomes are rare. If happens, in most cases spermatogenesis is severely affected, however, several men with this karyotype are fertile. Translocations between the X and Y chromosomes occur in several variations and often the karyotype is unbalanced. The correlation between karyotype and clinical presentation is complex. The phenotype may be male or female; fertility may be normal or it can be disturbed also.

The X chromosome contains numerous genes which are essential for survival. Every major deletion of this chromosome has a lethal effect in the male sex. Translocations between the X chromosome and an autosome usually result in disturbed spermatogenesis, whereas inversions of the X chromosome do not substantially affect male fertility.[96]

Y CHROMOSOME MICRODELETIONS

The human Y chromosome is the only dominant sex determinator, but plays an essential and important role in the genetic regulation of spermatogenesis. The long arm of the Y chromosome contains four partially overlapping but discrete regions that are essential for normal spermatogenesis. The loss of one of these regions, designated as *AZF*a (azoospermia factor a), *AZF*b (P5/proximal P1), *AZF*b (P5/distal P1) and *AZF*c (*b2/b4*), can lead to infertility. The deleted regions are usually of submicroscopic dimensions and are known as Y chromosome microdeletions. It can't be picked up by usual chromosomal analysis.[97, 98]

The prevalence of Y chromosome microdeletion in azoospermic men lies between 5-10% and between 2-5% in cases of severe oligozoospermia. Clearly, the frequency of Y microdeletions is related to the criteria by which men have been selected, but ethnic differences very well play an important role. Deletions of the *AZFc* region represent majority ~80% of all *AZF* deletions. The type and mechanism of deletions have been recently clarified and result from homologous recombination between retroviral or palindromic sequences.[99-103]

Clinically the patients present with severely affected spermatogenesis. But endocrine testicular function may or may not be affected by the microdeletion as in other cases of spermatogenetic failure. Testicular histopathology varies from complete or focal Sertoli-cell-only pattern to spermatogenic arrest or hypospermatogenesis with qualitatively intact but quantitatively severely reduced spermatogenesis. In azoospermic men, the presence of a complete deletion of *AZF*a or *AZF*b seems to be associated with uniform germ cell aplasia, negative prognostic value for testicular sperm retrieval and poor outcome. No clinical parameter can help distinguishing patients with microdeletions of the Y chromosome from infertile men without microdeletion. Y chromosome microdeletions are also seen in proven fertile men who have fathered pregnancies.[104-106]

A positive result of the analysis provides a causal explanation for the patient's disturbed spermatogenesis. Apart from this, the test also has prognostic value, as TESE is possible in about 50% of men with *AZF*c deletion and that paternal Y chromosome microdeletion is transmitted to every son of these patients and will probably inherit disturbed fertility. Genetic counseling

should be done for all carriers of Y chromosome microdeletions[107-112] (Figure 4.13).

Smaller deletions removing only part of the *AZFc* region have been identified as a polymorphism significantly associated with infertility, especially oligozoospermia. These are called *gr/gr* deletions occur by homologous recombination and have been extensively studied. They are found in about 6.8% of infertile men. But it is also seen in 3.9% of fertile men. Although they can act as a significant risk factor for male infertility, they should be regarded as a polymorphism and as of now the diagnosis offers no advantage in male infertility workup as no clinical consequence can be derived from the results obtained.[113-121]

VARICOCELE

The mechanism by which varicoceles cause infertility and the effectiveness of treatment in improving semen quality are still controversial.

Varicoceles are found in approximately 25% of men with infertility. An additional 15% may have a subclinical left varicocele which is detected only by valsalva maneuver or by scrotal Doppler study. Varicoceles are also seen in fertile men and are more common among tall men and in men with larger testes. They are rarely seen in men with severe testicular atrophy, e.g. in Kallmann and Klinefelter syndromes. When there is a moderate to large left varicocele, the left testis is usually smaller than the right testis (Figure 4.14).

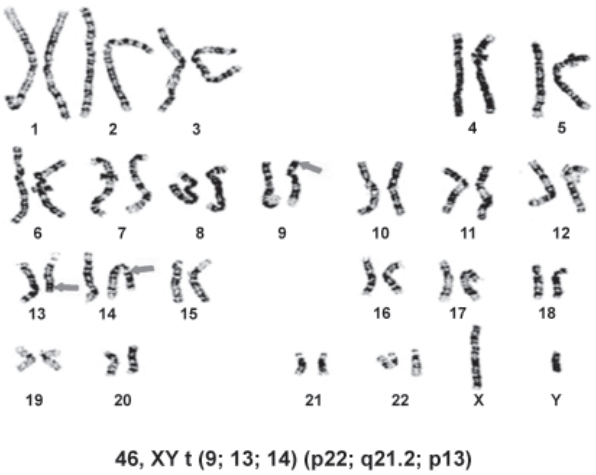


Figure 4.13: Y chromosome microdeletion

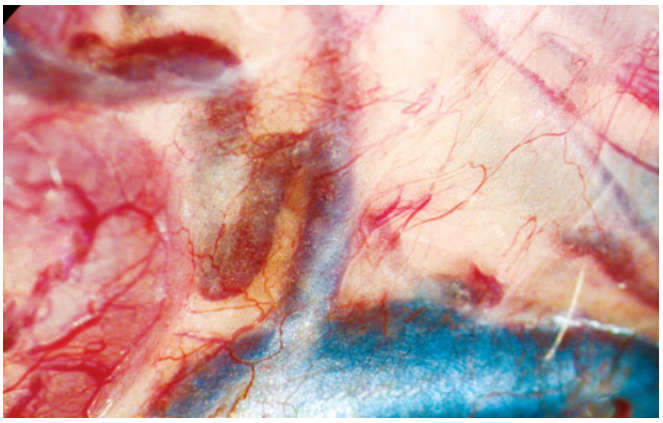


Figure 4.14: Laparoscopic view of varicocele

Pathophysiology

Men with varicoceles generally have poorer semen quality compared with men those who don't have varicocele. Thus, varicoceles can have an adverse effect on testicular function. Various theories have been proposed for these effects, which include vascular stasis, back pressure, interference with oxygenation, and reflux of renal or adrenal products into the pampiniform plexus, ROS generation and interference with the heat exchange function of the pampiniform plexus. Varicoceles are usually first noticed at puberty and thereafter may increase in size but remain relatively stable in size throughout the man's lifetime.

Most common symptoms include swelling and a dragging sensation in the scrotum, are infrequent, but many men with a large varicocele are unaware of its presence. The sudden appearance of a varicocele in an adult should be taken seriously because it may be a feature of a renal carcinoma with extension into the left renal vein.[122-126]

GENITAL TRACT OBSTRUCTION

Usually genital tract obstruction presents as azoospermia. But they have normal virilization, and normal serum FSH levels. However, some have combined obstruction and spermatogenic disorders or partial obstructions and severe oligospermia. There may be a past history of an incident that caused the obstruction, such as epididymitis with gonorrhea or associated respiratory disease. Because a few men with normal spermatogenesis have elevated FSH levels and some spermatogenesis may occur in association with a severe spermatogenic disorder, all patients should be offered further investigation.

One of the most common causes of genital tract obstruction is post vasectomy status. The presence of sperm antibodies in blood serum by indirect immunobead test indicates sperm are being formed but is an adverse prognostic factor for successful surgery.

Duration after surgery also gives an important prognostic clue for the success of further treatment. With bilateral congenital absence of the vasa or ejaculatory duct obstruction, semen volume, pH, and fructose levels are low. The semen does not have its characteristic smell and does not form a gel after ejaculation because it contains only prostatic and urethral fluid and does not contain seminal vesicle secretion. Rectal ultrasound shows absent or atrophic seminal vesicles with bilateral congenital absence of the vasa. But if the patient has ejaculatory duct obstruction, transrectal USG can show dilated seminal vesicles and ejaculatory ducts. Other one important cause of obstruction is cyst of the prostatic utricle. Testicular biopsy is usually normal but there may be some decline in spermatogenesis because of two reasons, as a coincidence or as a result of the obstruction, usually seen after vasectomy (Figure 4.15).

Pathophysiology

Usually degeneration of the Wolffian duct structures occurs with cystic fibrosis gene mutations but can be of different extent. Though most often only the head of the epididymidis is palpable, some men with bilateral congenital absence of the vasa have parts or all of the epididymides and scrotal vasa present with selective absence or atrophy of pelvic vasa and seminal vesicles.

Young's syndrome which is rare is not related to cystic fibrosis gene mutations. The pathology shows lipid inclusions in the epithelial cells and inspissated material in the head of the epididymis. Few men with this syndrome have fathered children, as the blockage develops in late adulthood.[127, 128]

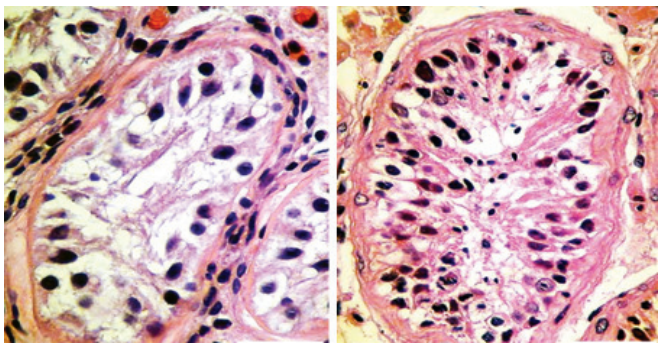


Figure 4.15: Comparison of histology of NOA and OA

Post-infectious inflammatory obstructions after gonorrhea typically occur in the tail of the epididymis, whereas nonspecific bacterial inflammation produces more widespread destruction. Tuberculosis usually causes multiple obstructions in the epididymides and vasa or destruction of the prostate and seminal vesicles. Back pressure blowout obstructions in the epididymis are usually seen after vasectomy. Many times iatrogenic injuries can also causes genital tract obstruction. Iatrogenic causes of geniital tract obstruction include accidental injury to epididymis during testicular biopsy, vasal damage during hernia repair or pelvic or lower abdominal surgery, especially of retroperitoneal area such as renal transplantation and ejaculatory duct obstruction from prostatectomy or complicated bladder catheterization.[129-131]

PATHOPHYSIOLOGY OF GERM CELL MALIGNANCY OF TESTES

The origin and pathogenesis of testicular germ cell tumors not clearly understood. There is an association with intrauterine factors and origin of germ cell tumors suggested by high incidence of testicular cancer in subjects with congenital errors of gonadal development and sexual differentiation. The neoplastic transformation of germ cells is initiated by factors acting *in utero*, usually in individuals with genetic susceptibility. Germ cell cancer precursor cells and primordial germ cells look very much similar and share some common distinct features, e.g. deficiency of intercellular bridges and a similar pattern of expression of various antigens. If aberrant development occurs, expressions of some of these antigens are totally changed. Recent studies done on the basis of regulation of the cell cycle in normal and neoplastic germ cells provided additional evidence demonstrating that germ cell cancer precursor cells have predominantly features of mitiotically dividing immature germ cells, so it is highly unlikely that they are derived from meiotic spermatocytes, as an alternative hypothesis stipulates[131-133] (Figure 4.16).

The exact mechanisms of neoplastic transformation of germ cells are not clearly understood. The hypothesis is that a disturbance in the fetal programming of gonadal development may be a result of an intrauterine hormonal imbalance, which is caused by a genetic disorder or by

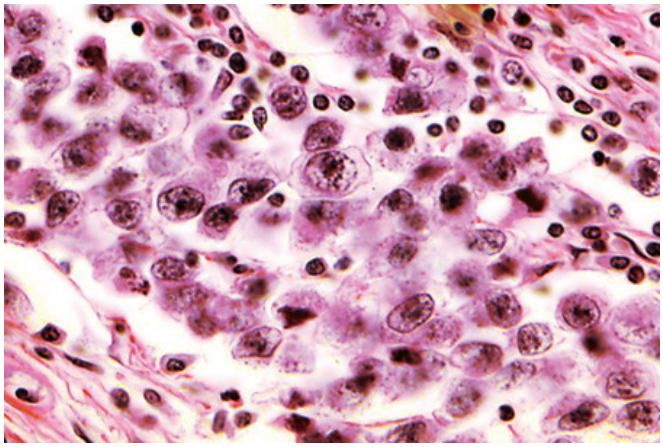


Figure 4.16: Histology of seminoma

an impact of an exogenous factor leading to a deficit of androgens or relative excess of estrogens.[134, 135]

Epidemiological factor influence, in particular the rising incidence of testicular cancer in developed countries, suggests a possible seminoma influence of environmental factors.

During the last few decades a great number of potent natural and synthetic hormones and hormone antagonists have been identified in environment which is otherwise known as "endocrine disrupting factors". Endocrine disrupting factors can cause a disturbance of hormonal milieu of the developing gonad and affect differentiation of early germ cells. This gave rise to a hypothesis that endocrine disrupters might have contributed to the reported decline in male reproduction mainly affecting semen parameters, and also acting as an etiological agent for testicular cancer development.[136 -141]

COITAL DISORDERS

Male coital disorders impacting fertility include erectile dysfunction (impotence), failure of ejaculation, retrograde ejaculation and premature ejaculation. Many men have problems with sexual performance after first learning about their infertility, but this usually ameliorates with time. Infrequent and poorly timed intercourse may result from incorrect advice, low libido, or the psychological reaction to infertility.[142]

Erectile Dysfunction

Erectile dysfunction may be associated with low libido from androgen deficiency with primary or secondary hypogonadism. Erectile dysfunction related to vascular or neurologic abnormalities (diabetic autonomic neuropathy or pelvic nerve damage) are uncommon in men presenting with infertility. Selective impotence at the time of ovulation may indicate psychological problems and ambivalence about having children.[143]

Failure of Ejaculation

Failure of ejaculation is usual with chronic spinal cord injury and may also be caused by antihypertensive and psychotropic drugs but otherwise is an infrequent cause of infertility in most societies. Healthy men who cannot ejaculate with intercourse may be able to produce semen by masturbation, with a vibrator, or other stimulation.[144,145]

Retrograde Ejaculation

Usually, there is a definite cause for retrograde ejaculation. They usually include—Prostatic surgery, diabetic neuropathy, pelvic nerve damage, or spinal cord injury. Retrograde ejaculation occurs when the bladder neck fails to contract at the time of ejaculation so that all or most of the semen passes into the bladder.

Often, a diagnosis of retrograde ejaculation can be made by taking an accurate history of previous surgical procedures and drug consumption. Retrograde ejaculation can be confirmed by demonstrating sperm presence in postcoital urine. A differential diagnosis should be pursued for patients who present with absent or low-volume ejaculates (such as an ejaculation, obstruction of an ejaculatory duct or seminal vesicle, or congenital anomalies of the accessory sex organs), with a complete physical examination and transrectal ultrasonography.[146]

Premature Ejaculation (PE)

Studies have been performed to investigate the etiology and pathogenesis of PE, but clear mechanisms have yet to be defined. The pathophysiology of PE can be differentiated into five distinct areas—psychorelational, neurobiological, urological, hormonal and andrological— which, consequently, suggests five different therapeutic approaches.[146]

According to the psychorelational theory, distortions of beliefs and false convictions about sexuality, established in childhood as a consequence of adverse influences on sexual behavior, might lead to sexual dysfunction such as PE.[147, 148]

Waldinger et al[149] have theorized, on the basis of animal models, that PE is not a psychological disorder but rather a neurobiological phenomenon due to chronic (genetic or acquired) central serotoninergic hypoactivity. Nevertheless, animal studies cannot be easily extrapolated to human sexual behavior, and further research is needed to clarify the role of the serotoninergic system in the pathogenesis of PE.

The uroandrological cause predisposes to the premature ejaculation is Chronic prostatitis, which is attributed as a cause in the recent time. Hormonal factor which causes premature ejaculation is hyperthyroidism. Three possible sites of action have been hypothesized: the sympathetic nervous system, the serotoninergic system and the endocrine/paracrine system In addition, thyroid hormone receptors have been identified in animal and human testes, and might also be present in other male genital tract structures that trigger ejaculation.[150, 151]

PATHOPHYSIOLOGY HYPOGONADOTROPIC HYPOGONADISM (HH)

The combinations of both blood sampling and genetic studies have contributed to the detailed understanding of the pathophysiology of isolated GnRH deficiency in the human beings. Direct sampling of GnRH is not possible in the human beings because it is exclusively confined to the hypophysio—portal system. So much so, because of short half-life of 2-4 min, it is not feasible to get an accurate measurement in the blood level. Because of these reasons always we should use other modalities to study GnRH secretion in the human beings.[152, 153]

PATTERNS OF GnRH SECRETION IN CASES WITH GnRH DEFICIENCY

A wide range of abnormalities in the GnRH secretion reflects the variety of clinical situations of Idiopathic HH (IHH). In IHH men, the spectrum of abnormalities in GnRH secretion or action is likely to contribute to the clinical and biochemical heterogeneity of this disorder.

Abnormal GnRH secretion patterns and their clinical presentations are in the following patterns.[154, 155]

1. Apulsatile form—Most severe form with no detectable LH pulses. Complete absence of pubertal development is noted.
2. Developmental arrest pattern—Predominant nocturnal LH pulses are noted and usual clinical manifestation is arrest of puberty.
3. Decreased amplitude pattern—LH pulses occurs at a normal frequency but were of diminished amplitude compared to those of normal individuals.
4. Normal pulsatality of LH with absent bioactivity.

COMPARISON OF ABNORMAL LH PULSATILITY AND CLINICAL FEATURES[156]

LH Pattern	*Testicular volume*	*Sperm concentration/ml*
Normal	~20 ml	70×10^6
Low amplitude	~5 ml	0×10^6
Developmental arrest	~8 ml	0×10^6
Apulsatile	~1 ml	0×10^6

IDIOPATHIC HYPOGONADOTROPIC HYPOGONADISM (IHH)

IHH is also known as congenital hypogonadotropic hypogonadism. There are 2 clinical types of IHH. They include

1. Complete IHH or Classical IHH
2. Partial IHH

   Partial IHH includes 3 subtypes of clinical syndromes. These are:

   a. *Adult onset IHH*—In this group of patients, puberty occurs normally and is followed years later by a decrease in libido, sexual function and fertility.

   b. *Fertile Eunuch Syndrome*—A patient with the fertile eunuch syndrome characterized by eunuchoidal proportions and lack of secondary sexual characteristics in the presence of normal size testes and preserved spermatogenesis.

   c. *Delayed puberty*—These patienta presents with a history of delayed but otherwise normal puberty among the families of patients with IHH.

Genetics of IHH

Wide variety of genetic heterogeneity has also been found in IHH. Most cases are sporadic and they comprise ~80%. This suggests that either the frequencies of spontaneous mutations are high or it may be stated that etiology of many cases may not be genetic. If we take the pattern of inheritance in familial cases, it includes autosomal dominant, autosomal recessive, or X-linked trait pattern of inheritance.[157] Kallmann's syndrome and IHH commonly shows with autosomal dominant inheritance with variable pentrance.[157, 158]

GENETIC RELATIONSHIPS OF HYPOGONADOTROPIC HYPOGONADISM

X-linked Genes

Kallmann's Syndrome Gene (KAL Gene)

Basic developmental defect in Kallmann's syndrome is the arrest of GnRH and olfactory neurons just below the telencephalon at the cribriform plate. Mutations in the KAL-1 gene were the first detected genetic association as the etiology of GnRH deficiency.[158, 159] Mutation in KAL gene causes defective production its protein product called Anosmin.[74] Thus mutations in the KAL gene cause premature stoppage of migration of olfactory and GnRH neurons to the brain causing anosmia and IHH. This defective GnRH neuronal migration results in the complete failure of activation of the hypothalamo-pituitary axis in patients with X-linked Kallmann's syndrome. Most X-linked Kallmann's syndrome mutations cause alteration of splicing, frameshift or stop codons and result in synthesis of a truncated anosmin protein. Though very rare missense mutations have also been described.[160, 161] X-linked Kallmann's syndrome patients present with the most severe form of IHH. Clinical features in these cases include an absolute lack of sexual development, an apulsatile pattern of GnRH-induced LH secretion, a high incidence of cryptorchidism and micropenis, very low inhibin B levels, and histological features consistent with immature testicular pattern.[162]

DAX-1 Gene

Defective DAX-1 gene (Xp21) encoding nuclear hormone receptor with a novel DNA-binding domain causes association of X-linked congenital adrenal hypoplasia with IHH.[163] Congenital adrenal hypoplasia shows a wide variety of clinical heterogeniety. It can vary from normal to complete absence of adrenal function. Many patients with congenital adrenal hypoplasia will respond abnormally to pulsatile GnRh therapy showing their defective development of hypothalamo-pituitary axis. So much so is the failure of hCG to induce normal spermatogenesis which shows the presence of an associated testicular defect.[164-166]

Autosomal Genes

The association of autosomal genes is noted most commonly in cases of familial hypogonadotropic hypogonadism. In these cases association with X-linked gene mutations are very less, which accounts only ~11% of cases.

GnRH Gene

Most clear and obvious autosomal candidate gene for GnRH deficiency is the GnRH gene itself, which is located at 8p21-8p11.2. There are no deletions, rearrangements and or point mutations have been described in the GnRH gene in human beings.[167-169]

GnRH-R Gene

The gene for the GnRH-R was recently cloned and its product is a G-protein-coupled receptor with seven transmembrane segments. Defects in the GnRH-R have recently emerged as the first autosomal cause of IHH. Activation of the GnRH-R results in increased activity of phospholipase C and mobilization of intracellular calcium by means of the Gq/G11 group of G proteins. While patients with a GnRH-R mutation were anticipated to manifest absolute hypogonadism and maximum unresponsiveness to GnRH stimulation. But milder variants of GnRH-R mutations have been described partially maintained GnRH resposiveness. These variable genotypes result in a wide phenotypical clinical spectrum ranging from the fertile eunuch syndrome to partial IHH to the most complete form of GnRH deficiency which has been discussed already.[170-174]

FGFR1 Gene

This is the most recently detected autosomal dominant gene for Kallmann's syndrome. Cleft palate/lip and dental agenesis are the two anomalies that are occasionally associated with KS. But these are the most consistent features noted commonly in patients with mutations in the FGFR1 gene. The presence of synkinesia, a phenotype previously considered to be specific for the X-linked form of KS, indicates that this neurological abnormality can occur also with autosomal transmission.[175]

GPR54 Gene

Invention of linkage analysis resulted in the identification of a novel locus for autosomal recessive IHH on 19p13. Sequencing of several genes located within this region showed that all affected siblings had a homozygous 155-bp deletion in GPR54, a G protein-coupled receptor gene.[176]

Genetic Defects of the Gonadotropins

Very rarely mutation in the Gene for LH-beta subunit and mutations in the Gene for FSH-beta subunit can also cause

IHH. In cases of LH subunit mutations, mutant hormone had no biologic activity but had normal immunoreactivity. Testosterone levels were reduced in association with elevated LH and FSH levels because of defective LH function. This clinical picture can easily be confused with primary hypogonadism, but these patients have a normal serum testosterone response to hCG administration. Features of FSH beta sub unit mutation are normal virilization, normal LH and testosterone levels, but small testes, azoospermia, and a high FSH level. But it has also been reported that these cases can have associated LH function abnormality also[177-179]

Hypogonadotropic Hypogonadism Associated with Other Pituitary Hormone Deficiencies

Combined pituitary hormone deficiency has been linked with rare abnormalities in genes encoding transcription factors necessary for pituitary development. Mutations in the gene, PROP-1 (Prophet of Pit-1) appear to be the most common cause of both familial and sporadic congenital combined pituitary hormone deficiency. The hormonal phenotype involves deficiencies of LH, FSH, GH, PRL, and TSH. Hypogonadism is a striking feature of patients with PROP-1 mutations, but there is variability in its clinical and hormonal expression. Most affected subjects fail to enter puberty and show consistently low LH and FSH responses to GnRH stimulation.[180]

PATHOPHYSIOLOGY OF ANTISPERM ANTIBODIES

The blood-testis barrier and the epididymal blood-epithelium barrier in humans are important structures in preventing sperm antigens from contacting immuno-competent cells, due to the tight junctions of Sertoli and epithelial cells.[181] This creates favorable conditions for spermatogenesis and sperm survival in the testicular fluid, and sperm maturation in the epididymal fluid. It also prevents the occurrence of autoimmunity after puberty. Therefore, alteration of the blood-testis barrier and the blood-epithelium barrier allows the production of ASAs and, hence, may lead to infertility.

Various protective mechanisms besides the blood-testis barrier and the blood-epithelium barrier have also been identified. Immunosuppressive substances have been found in semen and follicular fluid. Moreover, suppressor T lymphocytes in the human immune system, which partially mediate the normal state of immunologic unresponsiveness toward sperm autoantigens, also play an important role in preventing the autoimmune response. In addition, the cervix is a site of sperm filtration and uterine fluid has significantly high concentrations of IgG and IgA, which lead to increased phagocytosis of intact or defective sperm, and corrupted sperm debris. Moreover, components of seminal plasma and polymorphonuclear neutrophils in semen could eliminate nonviable sperm or debris[182]

Recently, three complement-regulatory proteins, decay-accelerating factor, membrane cofactor protein and CD59, have been found on spermatozoa and also in the extrafetal tissues.[183] It is likely that these inhibitors are essential for normal reproductive function.

A soluble form of Fc γ RIII (CD16) has been isolated from seminal plasma, which may modulate immunosuppression of antisperm immune responses in both male and female reproductive tracts. Individuals who were ASA positive had lower detectable levels of Fc γ RIII compared with those who were ASA negative, indicating that the inhibition of CD16 plays an important role in preventing ASA production.[184]

Factors Causing to ASA Production

During maturation of spermatogenesis, new antigens are expressed on developing spermatocytes and spermatids. When these antigens come into contact with immuno-competent cells, or spermatozoal antigens are exposed to the mucosal and systemic immune systems, ASA formation occurs. Developmental abnormalities of the formation of the blood-testis barrier, traumatic disruption, infection or unilateral focal cryptic obstructions could lead to immunogenic sperm antigens being exposed to the immune system thus initiating an immune response to produce ASAs.[185] Gastrointestinal exposure to sperm was also associated with the development of ASAs in homosexual men. In addition, in some cases, immunity to sperm cells may be the result of altered sperm antigens.

Chronic Infections

A chronic infection, such as *Chlamydia trachomatis*, which is capable of eliciting an immune response, has been demonstrated in male and female genital tracts. Several authors reported that Hsp60 in human seminal fluid was associated with the formation of ASAs. Heat-shock protein (Hsp) 60 is associated with a humoral immune response to *C. trachomatis* or other infection agents,

indicating that genital infection could be an important factor causing ASA production.[186]

The possible relationship between ASA production and *Ureaplasma urealyticum* infection was also investigated. It was confirmed that *U. urealyticum* and human sperm membrane proteins share cross-reactive antigens (61, 50 and 25 kDa). Among the cross-reactive antigens, the urease complex component UreG of *U. urealyticum* was determined.[187]

SURGERIES ON VAS DEFERENS (VASECTOMY AND VASOVASOSTOMY)

Vasovasostomy has become a popular and highly successful method for restoring fertility to those who have undergone a vasectomy. It was reported that there was a high correlation between vasostomy and ASA production. More than 50-70% of men develop ASAs after a vasectomy and there is limited success in the regain of fertility, even after successful surgical reanastomosis in vasovasostomy attributed to the presence of ASAs. The highest incidence of titers is 1 year after vasectomy, but titers can be found as early as 6 months or as late as 20 years postoperatively. These ASAs are causative factors of infertility, because disappearance of ASAs causes regain of fertility.[188]

However, considerable disagreement remains as to whether antibodies are the primary causative agent. No significant changes were observed in the prevalence of the antibodies over the period following vasectomy, and in patients with and without postoperative sperm granulomas. Sperm antigens are in abundant supply in vasectomized men as a result of the continuous resorption of spermatozoa after vasectomy. Therefore, it is possible that undetectable antibody titers reflect high levels of ASAs circulating as immune complexes.[189]

Other Factors

Heavy metals can also adversely influence reproduction since in sensitive individuals they are able to alter the immune responses including autoantibody production, this can then cause infertility. For example, after mercury stimulation, less IFN-γ and more ASAs were produced by the lymphocytes of patients. This suggested that the release of metal ions from dental materials can be one of the stimulating factors that may adversely affect fertility.[190, 191]

Testicular cancer and testes torsion patients also have a markedly high incidence of ASAs, which may be related to radiation therapy and severe damage of seminiferous tubules. However, it was observed that no rabbits had detectable levels of antisperm IgG after testes torsion compared with controls. In addition, a high ASA rate (43.1%) was observed among prostitutes. It may be related to repeated inoculations with multiple sperm antigens and/or microorganisms.[192, 193]

Mechanisms of ASA-induced Infertility

Immunologic infertility is an end result of the combined actions of multiple ASAs. ASAs can be present at different sites, can react against different antigens, and can impair fertility in various ways. ASAs can negatively affect sperm motility, cervical mucus penetration, the acrosome reaction, zona pellucida recognition and the fusion of gametes, by immobilizing and/or agglutinating sperm, blocking sperm-egg interaction, preventing implantation, and/or arresting embryo development in the pre-implantation period. The ASAs have functional significance only when they are fixed to their antigens. ASA directed against the sperm head were of prime importance because it can interfere with fertilization, whereas those against the tail were involved in causing poor motility. Moreover, ASA-coated sperm may be more vulnerable to phagocytosis in the female reproductive tract.[194]

ASA INDUCED DEFECTIVE MOTILITY OF SPERM

Impairement of sperm motility were the main cause of infertility in many patients, resulting in defective transport in female genital tract and inability of union with the oocyte. ASAs can bind to antigens of sperm membranes, but it can never reach subcellular structures living cells. Therefore, it is speculated that they alter function of transmembrane proteins and they can also cause complement-mediated membrane damage finally causing impaired motility of sperm.

Complement-dependent inactivation of sperm motility might be the biological mechanism of female infertility. IgG- and C3-bound motile sperm can adhere to human neutrophils *in vitro* and the adhesion occur at the acrosome region of sperm head. This adhesion initiates the localized release of oxygen radicals at the site of neutrophil/sperm membrane contact. The

deposition of activated C3 fragments, the assembly of terminal membrane attack complexes (C5b-9) and oxygen radicals could lead to C3-mediated sperm binding to neutrophils or C5b-9-mediated sperm-motility loss.[195,196]

Compliment fixation of sperm causes marked neutrophil aggregation and expression of CD11b on the surface of these neutrophils which accelerates sperm phagocytosis. CD11b upregulation correlated with a significant increase in tyrosine phosphorylation of neutrophil proteins during sperm phagocytosis.

ASA AND SPERM PENETRATION OF CERVICAL MUCUS

The presence of ASAs in cervical secretions is not very common but when present it can cause severe form of infertility. ASAs influences sperm penetration of cervical mucus by the immobilization and shaking phenomenon of sperm in cervical mucus. Surface antigens of the acrosome and sperm tail principal piece appear to be recognized by circulating sperm-immobilizing and -agglutinizing antibodies.

Impairment of sperm penetration into the cervical mucus appears to be of consequence of the activation of the complement cascade by immunoglobulins attached to the sperm surface, at the end of which cell lysis and initiation of the phagocytotic process take places. The complement induced cell lysis depends on the immunoglobulin class of the antibody. IgM is far more effective than IgG, while some IgA subclasses are unable to interact with the early complement components. During their residence in the cervical mucus, sperm are exposed to complement activity. The complement activity in cervical mucus is less and only approximately 12% of activity of serum. So it may take longer for sperm immobilization to occur in the cervical mucus.

Another mechanism is the binding occurring between the Fc region of the antibody and the cervical mucus. This process will contribute to the binding of spermatozoa to the antibody and the immobilization of this complex by the cervical mucus that is also seen in immunological infertility.[197-199]

ASA ACTION ON ACROSOME REACTION (AR)

There is strong evidence to suggest that the antigens involved in the AR and antibodies to these antigens can play a role in immunological infertility. ASA binding to the sperm head influences the acrosome reaction. A number of spontaneously occurring ASAs were shown to enhance the number of acrosome-reacted sperm. ASAs or antigens inhibiting the spontaneous AR include antiactin antibody, TSA-1, FA-1 and calpastatin. These can impair acrosome reaction and exert their effect on immunological infertility.[198, 199]

MONONUCLEAR CELLS AND IMMUNOLOGICAL INFERTILITY

Mononuclear cells include lymphocytes, monocytes and macrophages, which may play a role in infertility caused by ASAs. Cytokines secreted by these mononuclear cells may also have role, but these are unclear at present and still in the hypothesis level.

Antibody-coated sperm will activate lymphocytes, thus leading to the production of ASAs. This may be an additional mechanism that leads to infertility. T lymphocytes bearing the αβ- and γδ-antigen receptors were present in semen samples. The presence of ASA was associated with elevated concentrations of both γδ and αβ T lymphocyte cells. The αβ and/or γδ T lymphocytes and/or monocytes/macrophages in semen act as a source of Hsp60 (Heat Shock Protein 60). Major source of Hsp 60 is presumed to be the γδ T lymphocytes. On the contrary, γδ T cells can induce Hsp60 expression in these cells also. Thus, the interaction of T lymphocytes with Hsp60 may be responsible for ASA production.[200, 201]

However, not all men with ASAs were positive for seminal Hsp60. It has been reported that no significant difference was found in the concentration of leukocytes or subpopulations of these cells (monocytes, lymphocytes and granulocytes) between fertile, sterile without ASAs and immunological sterile groups. These contradictory data indicated that the association between chronic infection of the male genital tract, γδ T-cell activation, antisperm autoimmunity and Hsp60 expression requires further exploration. It is also possible that both the presence of ASAs and the increase of leukocytes in semen are manifestations of an immunological response or a common etiological factor of genital tract infections, and they are not interrelated.[202]

In addition, a significant increase in large granular lymphocytes (LGL) was detected in semen samples from immunologically sterile patients. Immuno-regulation by Antibody Dependent Cellular Cytotoxicity (ADCC) induces the disappearance of the antigens recognized by

each antibody. Thus, the appearance of ASAs in an ejaculate could generate the expansion and activation of LGLs to decrease ASA production by B lymphocytes. Nevertheless, LGL activity can be a specific cellular mechanism executed in checking the fertilizing capacity of these spermatozoa.[203, 204]

Antisperm cell-mediated immunity (CMI) has been associated with infertility in men and women. Sperm antigens can specifically induce CMI factors that have detrimental effects on sperm motility and also known to have deleterious effect on preimplantation embryos. Lymphokines and monokines can affect the fertilizing ability of human spermatozoa in the zona-free hamster egg-penetration test, indicating that soluble factors produced by activated lymphocytes and macrophages in reproductive tissues could be significant mediators of immunologic infertility.[206] However, antisperm CMI responses have been observed in all individuals of the following groups: infertile men and women, fertile women, virgin women, oligozoospermic men, and men with andrological disorders. Therefore, the role of antisperm CMI in reproductive processes also remains enigmatic.[207]

In general, mechanisms by which ASAs inhibit fertility are still unclear and can be unique to each individual's antibodies. ASA immunization has only a relative effect on impairment of fertilization and never causes an absolute effect. As per our current knowledge we should think that the interference of ASAs at the level of gamete interaction is more important than that at any other levels. As of now, interaction at the gamete level can be negotiated by doing ICSI.[208]

OXIDATIVE STRESS AND MALE INFERTILITY

What is Oxidative Stress and how does it Affect Fertility?

Of late many studies in the area of male infertility have been focused on oxidative stress-related mechanisms of sperm damage and their role in male infertiltiy. Human spermatozoa are capable of generating controlled low amounts of endogenous ROS that play a significant role in inducing sperm capacitation/acrosome reaction and acquisition of fertilization. But when it goes to a pathological level it can adversely affect the sperm and contribute to the damage to the spermatozoa.[209]

Normally as it happens every where in the body, there exists a balance between free radical generating and scavenging systems in male reproductive tract also. However, high generation of ROS by immature and abnormal spermatozoa, contaminating leukocytes, by sperm processing (e.g., excessive centrifugation, cryopreservation/thawing), accompanied by low scavenging and antioxidant levels in serum, seminal plasma, and/or sperm-processing media can induce a state of relative oxidative stress. High levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) endanger sperm motility, viability, and function by interacting with membrane lipids, proteins, and nuclear and mitochondrial function.[210]

The presumed explanation for this damaging process is based on the fact that mammalian spermatozoa membranes are rich in polyunsaturated fatty acids (PUFA) that make them very fluid but at the same time very susceptible to free radical-induced peroxidative membrane damage. Membrane fluidity is essentially needed to support the membrane fusion events associated with a variety of secretory episodes. In the case of spermatozoa, it facilitates the acrosome reaction and the sperm-egg fusion during fertilization.[211, 212]

OXIDATIVE STRESS AND SPERM MEMBRANE LIPID PEROXIDATION

Spermatozoa, unlike other cells, are unique in structure, function, and very susceptible to damage by ROS. Orientation of polyunsaturated fatty acids (PUFA) in the plasma membrane creates the fluidity necessary by the spermatozoa to perform normal physiological functions like acrosome reaction and sperm-oocyte fusion. Also, membrane-bound ATPases that function as ion pumps help maintain the correct intracellular concentrations of nutrients and ions especially sodium and calcium, are critically dependent on membrane fluidity. If the latter is lost, then the ATPases cease to function and the cells suddenly start to accumulate these ions at a rate that ultimate leads to their destruction.[210, 211]

In general, the most significant effect of membrane lipid peroxidation (LPO) in all cells is the perturbation of membrane structure and its function (Figure 4.17). Low levels of NADH and glutathione, as a result of the increased activity of glutathione peroxidase to remove metabolites of LPO, will further affect cellular Ca^{2+} homeostasis. Minor alterations in sperm membranes in selected cases of oligo-asthenozoospermia can be reversed by glutathione (GSH) therapy.[212-215]

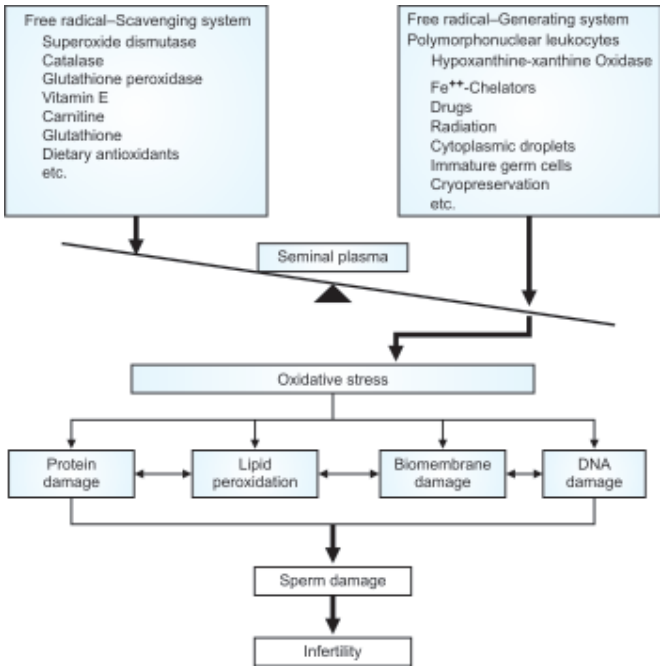


Figure 4.17: Pathogenesis of infertility in oxidative stress

Oxidative Stress and Protein Damage

Besides affecting membrane components and fluidity, ROS-induced peroxidation of critical thiol groups in proteins will alter structure and function of spermatozoa and ova with an increased susceptibility to attack by macrophages.[216] In addition, the redox status of human spermatozoa is likely to affect phosphorylation and adenosine triphosphate (ATP) generation with a profound influence on its fertilizing potential.[217, 218] In general, the oxidizing conditions increase tyrosine phosphorylation with enhanced sperm function, whereas reducing conditions have the opposite negative effect. Similarly, ROS-induced protein glycosylation may also contribute to such damage.[219-224]

Peroxidative Damage to Spermatozoa

Peroxidative damage initiated by high ROS generation during oxidative stress as seen in the spermatozoa of infertile men is associated with not only a loss of membrane function, but also the appearance of damage to the DNA located in the sperm head in such patients, leading to a high incidence of DNA strand breaks. Under normal circumstances, spermatozoa with damaged DNA would not participate in the fertilization process because of collateral peroxidative damage to the sperm plasma membrane. This peroxidation cascade gets stimulated by the presence of transition metals such as iron to form initiating species (L) that decompose membrane lipid peroxides.[213-215]

Oxidative Stress and Apoptosis in Sperm DNA Damage

Current concepts on oxidative stress suggest damage to integrity of DNA in the sperm nucleus resulting in base modification, DNA fragmentation and chromatin cross-linking. Such DNA damage may accelerate the process of germ cell apoptosis. In the testis, that can lead to a decline in sperm counts and result in infertility.[231-233] Recent studies have indicated a significant increase in the levels of apoptotic spermatozoa in the semen of infertile men. Patients who were inseminated with samples containing higher degrees of DNA damage (>15%) had poor embryo quality and/or experienced miscarriages. The oxidative damage to mitochondrial DNA (mt DNA) is also known to occur in all aerobic cells that are rich in mitochondria including spermatozoa.[225-227] Multiple mt DNA deletions in spermatozoa could arise through a free radical-driven event occurring at the spermatogonial cell stage and can account for reproductive failure in some men. Although standard sperm parameters are not predictive of high levels of apoptosis, it may be an independent phenomenon that plays an important role in the pathophysiology of male infertility.[228-234]

REFERENCES

1. Clermont Y, Huckins C. Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male albino rats. Am J Anat 1961;108:79-97.
2. de Kretser DM, Kerr JB. The cytology of the testis. In Knobil E, Neill JD (eds). The Physiology of Reproduction, 2nd ed. New York, Raven Press, 1994;1177-1290.
3. Meistrich ML, van Beek M. Spermatogonial stem cells. In Desjardins C, Ewing LL (eds). Cell and Molecular Biology of the Testis. New York, Oxford University Press, 1993;266-95.
4. Clermont Y. Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 1972;52:198-236.
5. Clermont Y. Two classes of spermatogonial stem cells in the monkey (Cercopithecus aethiops). Am J Anat 1969;126:57-71.
6. van Alphen MM, van de Kant HJ, de Rooij DG. Depletion of the spermatogonia from the seminiferous epithelium of the rhesus monkey after X irradiation. Radiat Res 1988;113:473-86.
7. Schlatt S, Weinbauer GF. Immunohistochemical localization of proliferating cell nuclear antigen as a tool

to study cell proliferation in rodent and primate testes. Int J Androl 1994;17:214-22.
8. Schulze C. Morphological characteristics of the spermatogonial stem cells in man. Cell Tissue Res 1979;198:191-9.
9. Dym M, Fawcett DW. Further observations on the numbers of spermatogonia, spermatocytes and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 1971;4:195-215.
10. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci USA 2000;97:8346-51.
11. Stubbs L, Stern H. DNA synthesis at selective sites during pachytene in mouse spermatocytes. Chromosoma 1986;93:529-36.
12. Clermont Y. Kinetics of spermatogenesis in mammals. Seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 1972;52:198-236.
13. Heller CG, Clermont Y. Kinetics of the germinal epithelium in man. Rec Prog Horm Res 1964;20:545-75.
14. Dobson MJ, Pearlman RE, Karaiskakis A, et al. Synaptoneural complex proteins: occurrence, epitope mapping and chromosome dysfunction. J Cell Sci 1994;197:2749-60.
15. Menwissen RLJ, Offenberg HH, Dietrich AJJ, et al. A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes. EMBO J 1992;11:5091-5100.
16. Morelli MA, Cohen PE, Not all germ cells are created equal: Aspects of sexual dimorphism in mammalian meiosis. Reproduction 2005;130:761-81.
17. de Kretser DM. Ultrastructural features of human spermiogenesis. Z Zellforsch 1969;98:477-505.
18. Jegou B, Laws AO, de Kretser DM. Changes in testicular function induced by short-term exposure of the rat testis to heat: further evidence for interaction of germ cells, Sertoli cells and Leydig cells. Int J Androl 1984;7:244-57.
19. Lin D, Sugawara T, Strauss III JF, et al. Role of Steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 1995;267:1828-31.
20. Morse HC, Horike N, Rowley MJ, Heller CG. Testosterone concentrations in testes of normal men: effects of testosterone propionate administration. J Clin Endocrinol Metab 1973;37:882-6.
21. Mc Lachlan RI, O'Donnell L, Stanton PG, et al. Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones and germ cell populations in normal young men. J Clin Endocrinol Metab 2002;87:546-56.
22. Meachem SJ, Wreford NG, Stanton PG, Robertson DM, McLachlan RI. Follicle-stimulating hormone is required for the initial phase of spermatogenic restoration in adult rats following gonadotropin suppression. J Androl 1998;19:725-35.
23. Russell LD, Kershaw M, Borg KE, El Shennawy A, Rulli SS, Gates RJ, Calandra RS. Hormonal regulation of spermatogenesis in the hypophysectomized rat: FSH maintenance of cellular viability during pubertal spermatogenesis. J Androl 1998;19:308-19.
24. McLachlan RI, Wreford NG, Meachem SJ, De Kretser DM, Robertson DM. Effects of testosterone on spermatogenic cell populations in the adult rat. Biol Reprod 1994;51:945-55.
25. Dym M, Fawcett DW. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium Biol Reprod 1970;3:308-26.
26. J Ãgou B. The Sertoli cell. Ballieres Clin Endo Metab 1992;6:273-311.
27. 61. Hall PF, Mita M. Influence of FSH on glucose transport by cultured Sertoli cells. Biol Reprod 1984;31:863-69.
28. Jutte NHPM, Hansen R, Grottegoed JA, et al. FSH stimulation of the production of pyruvate and lactate by rat Sertoli cells maybe involved in hormonal regulation of spermatogenesis. J Reprod Fert 1983;68:219-26.
29. Morales C, Sylvester SR, Griswold MD. Transport of iron and transferrin synthesis by the seminiferous epithelium of the rat in vitro. Biol Reprod 1987;37:995-1005.
30. Skinner MK, Griswold MD. Sertoli cells synthesize and secrete a ceruloplasmin-like protein. Biol Reprod 1983;28:1225-9.
31. Sharpe RM. Intratesticular factors controlling testicular function. Biol Reprod 1984;30:29-49.
32. Simorangkir DR, Wreford NG, de Kretser DM. Impaired germ cell development in the testis of immature rats with neonatal hypothyroidism. J Androl 1997;18:186-93.
33. Boitani C, Stefanini M, Fragale A., Morena AR. Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. Endocrinology 1995;136:5438-44.
34. Meehan T, Schlatt S, O'Bryan MK., de Kretser DM, Loveland KL. Regulation of germ ell and Sertoli cell development by activin, follistatin and FSH. Development 2000;220:225-37.
35. Cortes D, Muller J, Skakkebaek NE. Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. Int J Androl 1987;10:589-96.
36. Sheckter CB, McLachlan RI, Tenover JS, et al. Serum inhibin concentrations rise during GnRH treatment of men with idiopathic hypogonadotrophic hypogonadism. J Clin Endocrinol Metab 1988;67:1221-24.
37. de Kretser DM. Local regulation of testicular function. Int Rev Cytol 1987;10:89-112.
38. Loveland KL, Schlatt S. Stem cell factor and c-kit in the mammalian testis: Lessons originating from Mother Nature's gene knock-outs. J Endocrinol 1997;153:337-44.
39. Merry C, Sweeney B, Puri P. The vanishing testis: anatomical and histological findings. Eur Urol 1997;31:65-7.
40. Smith NM, Byard RW, Bourne AJ. Testicular regression syndrome – a pathological study of 77 cases. Histopathology 1991;19:269-72.
41. Davenport M, Brain C, Vandenberg C, Zappala S, Duffy P, Ransley PG, Grant D. The use of the hCG stimulation test in the endocrine evaluation of cryptorchidism. Br J Urol 1995;76:790-94.

42. Lee MM, Donahoe PK, Silverman BL, Hasegawa T, Hasegawa Y, Gustafson ML, Chang Y, MacLaughlin DT. Measurements of serum Müllerian inhibiting substance in the evaluation of children with nonpalpable gonads. N Engl J Med 1997;336:1480-86.
43. Misra M, MacLaughlin DT, Donahoe PK, Lee MM. Measurement of Mullerian inhibiting substance facilitates management of boys with microphallus and cryptorchidism. J Clin Endocrinol Metab 2002;87:3598-602.
44. Nieschlag E, Nieschlag S, Behre HM. Lifespan and testosterone. Nature 1993;366:215.
45. Kremer H, Kraaij R, Toledo SP, Post M, Fridman JB, Hayashida CY, van Reen M, Milgrom E, Ropers HH, Mariman E, Themmen APN, Brunner HG. Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene. Nat Genet 1995;9:160-4.
46. Martens JW, Lumbroso S, Verhoef-Post M, Georget V, Richter-Unruh A, Szarras-Czapnik M, Romer TE, Brunner HG, Themmen AP, Sultan Ch. Mutant luteinizing hormone receptors in a compound heterozygous patient with complete Leydig cell hypoplasia: abnormal processing causes signalling deficiency. J Clin Endocrinol Metab 2002;87:2506-13.
47. Gromoll J, Eiholzer U, Nieschlag E, Simoni M. Male hypogonadism caused by a homozygous deletion of exon 10 of the luteinizing hormone receptor. J Clin Endocrinol Metab 2000;85:2281-86.
48. Müller T, Gromoll J, Simoni M. Absence of exon 10 of the human luteinizing hormone (LH) receptor impairs LH, but not human chorionic gonadotropin action. J Clin Endocrinol Metab 2003;88:2242-49.
49. Del Castillo EB, Trabucco A, de la Balze A. Syndrome produced by absence of the germinal epithelium without impairment of the Sertoli or Leydig cells. J Clin Endocr 1947;7:493.
50. Silber SJ, Van Steirteghem AC, Devroiy P. Sertoli cell only revisited (editorial). Hum Reprod 1995;10:1031-2.
51. Foresta C, Ferlin A, Garollo A, Moro E, Pistorello M, Barbaux S, Rossato. High frequency of well-defined Y chromosome deletions in idiopathic Sertoli cell-only syndrome. Hum Reprod 1998;13:302-7.
52. Schulze W, Thoms F, Knuth UA. Testicular sperm extraction: comprehensive analysis with simulataneously performed histology in 1418 biopsies from 766 subfertile men. Hum Reprod 1999;14:82-96.
53. McLachlan R, Rajpert-De Meyts Ewa, Christina E Hoei-Hansen CE, de Kretser DM, Skakkebaek NE. Histological evaluation of the human testis: approaches to optimizing the clinical value of the assessment Human Reproduction 2007;22:2-16.
54. Weinbauer GF, Behr R, Bergmann M, Nieschlag E. Testicular cAMP responsive element modulator (CREM) protein is expressed in round spermatids but is absent or reduced in men with round spermatid maturation arrest. Mol Hum Reprod 1998;4:9-15.
55. Nishimune Y, Tanaka H. Infertility caused by polymorphisms or mutations in spermatogenesis-specific genes. J Androl 2006;27:326-34.
56. Tüttelmann F, Rajpert-De Meyts E, Nieschlag E, Simoni M. Gene polymorphisms and male infertility – a meta-analysis and literature review. RBM Online 2007;15:643-58.
57. Davis-Dao CA, Tuazon ED, Sokol RZ, Cortessis VK. Male infertility and variation in CAG repeat length in the androgen receptor gene: a meta-analysis. J Clin Endocrinol Metab 2007;92:4319-26.
58. Matin-du-Pan RC, Campana A. Physiopathology of spermatogenic arrest. Fertil Steril 1993;60:937-46.
59. Foresta C, Bettella A, Merico M, Garolla A, Plebani M, Ferlin A, Rossato M. FSH in the treatment of oligozoospermia. Mol Cell Endocrinol 2000;161:89-97.
60. Bergmann M, Behre HM, Nieschlag E. Serum FSH and testicular morphology in male infertility. Clin Endocrinol 1994;40:133-6.
61. Ballesca JL, Balasch J, Calafell JM, Alvarez R, Fabregues F, de Osaba MJ, Ascaso C, Vanrell JA. Serum inhibin B determination is predictive of successful testicular sperm extraction in men with non-obstructive azoospermia. Hum Reprod 2000;15:1734-8.
62. Brugo-Olmedo S, De Vincentiis S, Calamera JC, Urrutia F, Nodar F, Acosta AA. Serum inhibin B may be a reliable marker of the presence of testicular spermatozoa in patients with nonobstructive azoospermia. Fertil Steri 2001;76:1124-9.
63. von Eckardstein S, Simoni M, Bergmann M, Weinbauer GF, Gassner P, Schepers AG, Nieschlag E. Serum inhibin B in combination with serum follicle-stimulating hormone (FSH) is a more sensitive marker than serum FSH alone for impaired spermatogenesis in men, but cannot predict the presence of sperm in testicular tissue samples. J Clin Endocrinol Metab 1999;84:2496-2501.
64. Bohring C, Schroeder-Printzen I, Weidner W, Krause W. Serum levels of inhibin B and follicle-stimulating hormone may predict successful sperm retrieval in men with azoospermia who are undergoing testicular sperm extraction. Fertil Steril 2002;78:1195-8.
65. Kamischke A, Behre HM, Bergmann M, Simoni M, Schafer T, Nieschlag E. Recombinant human follicle stimulating hormone for treatment of male idiopathic infertility: a randomized, double-blind, placebo-controlled, clinical trial. Hum Reprod 1998;13:596-603.
66. Klinefelter HF, Reifenstein EC, Albright F. Syndrome characterized by gynecomastia, aspermatogenesis without A-Leydigism, and increased excretion of follicle stimulating hormone. J Clin Endocrinol 1942;2:615-27.
67. Bojesen A, Juul S, Gravholt CH. Prenatal and postnatal prevalence of Klinefelter syndrome: A national registry study. J Clin Endocrinol Metab 2003;88:622-6.
68. Morris JK, Alberman E, Scott C, Jacobs P. Is the prevalence of Klinefelter syndrome increasing? Eur J Hum Genet 2008;16:163-70.

69. Ottesen AM, Garn ID, Aksglaede L, Juul A, Rajpert-De Meyts E. A simple screening method for detection of Klinefelter syndrome and other X-chromosome aneuploidies based on copy number of the androgen receptor gene. Mol Hum Reprod 2007;13:745-50.
70. Thomas NS, Hassold TJ. Aberrant recombination and the origin of Klinefelter syndrome. Hum Reprod Update 2003;9:309-17.
71. Westlander G, Ekerhovd E, Bergh C. Low levels of serum inhibin B do not exclude successful sperm recovery in men with nonmosaic Klinefelter syndrome. Fertil Steril. 2003;79 Suppl 3:1680-82
72. Kamischke A, Baumgardt A, Horst J, Nieschlag E. Clinical and diagnostic features of patients with suspected Klinefelter syndrome. J Androl 2003;24:41-8.
73. Christiansen P, Andersson AM, Skakkebaek NE. Longitudinal studies of inhibin B levels in boys and young adults with Klinefelter syndrome. J Clin Endocrinol Metab 2003;88:888-91.
74. Hasle H, Mellemgaard A, Nielsen J, Hansen J. Cancer incidence in men with Klinefelter syndrome. Br J Cancer 1995;71:416-20.
75. Giordano SH, Buzdar AU, Hortobagyi GN. Breast cancer in men. Ann Intern Med 2002;137:678-87.
76. Samango-Sprouse C. Mental development in polysomy X Klinefelter syndrome (47, XXY; 48, XXXY): effects of incomplete X inactivation. Semin Reprod Med 2001;19:193-202.
77. Ratcliffe SG. The psychological and psychiatric consequences of sex chromosome abnormalties in children, based on population studies. In Basic approaches to genetic and molecularbiolological developmental psychiatry. Poustka F (ed) Quintessenz Berlin, 1994;99-122.
78. Rovet J, Netley C, Bailey J, Keenan M, Stewart D. Intelligence and achievement in children with extra X aneuploidy: A longitudinal perspective. Am J Med Genet (Neuropsychiatric Genetics) 1995;60:356-63.
79. Zitzmann M, Depenbusch M, Gromoll J, Nieschlag E. X-chromosome inactivation patterns and androgen receptor functionality influence phenotype and social characteristics as well as pharmacogenetics of testosterone therapy in Klinefelter patients. J Clin Endocrinol Metab 2004;89:6208-17.
80. Terzoli G, Simoni G, Lalatta F, Colucci G, Lobbiani A. Fertility in a 47, XXY patient: assessment of biological paternity by deoxyribonucleic acid fingerprinting. Fertil Steril 1992;58:821-25.
81. Foresta C, Galeazzi C, Bettella A, Stella M, Scandellari C. High incidence of sperm sex chromosomes aneuploidies in two patients with Klinefelter's syndrome. J Clin Endocrinol Metab 1998;83:203-5.
82. Rives N, Joly G, Machy A, Simeon N, Leclerc P, Mace B. Assessment of sex chromosome aneuploidy in sperm nuclei from 47, XXY and 46, XY/47, XXY males: comparison with fertile and infertile males with normal karyotype. Mol Hum Reprod 2000;6:107-12.
83. Bergere M, Wainer R, Nataf V, Bailly M, Gombault M, Ville Y, Selva J. Biopsied testis cells of four 47, XXY patients: fluorescence in-situ hybridization and ICSI results. Hum Reprod 2002;17:32-37.
84. Morel F, Bernicot I, Herry A, Le Bris MJ, Amice V, De Braekeleer M. An increased incidence of autosomal aneuploidies in spermatozoa from a patient with Klinefelter's syndrome. Fertil Steril 79 Suppl 2003;3:1644-46.
85. Madgar I, Dor J, Weissenberg R, Raviv G, Menashe Y, Levron J. Prognostic value of the clinical and laboratory evaluation in patients with nonmosaic Klinefelter syndrome who are receiving assisted reproductive therapy. Fertil Steril 2002;77:1167-69.
86. Yamamoto Y, Sofikitis N, Mio Y, Loutradis D, Kaponis A, Miyagawa I. Morphometric and cytogenetic characteristics of testicular germ cells and Sertoli cell secretory function in men with non-mosaic Klinefelter's syndrome. Hum Reprod 2002;17:886-96.
87. Staessen C, Tournaye H, Van Assche E, Michiels A, Van Landuyt L, Devroey P, Liebaers I, Van Steirteghen A. PGD in 47, XXY Klinefelter's syndrome patients. Hum Reprod Update 2003;9:319-30.
88. Nielsen J, Pelsen B, Sornensen K. Follow-up of 30 Klinefelter males treated with testosterone. Clin Genet 1988;33:262-69.
89. De la Chapelle A, Koo GC, Wachtel SS. Recessive sex-determining genes in human XX male syndrome. Cell 1978;15:837-42.
90. Fechner PY, Marcantonio SM, Jaswaney V, Stetten G, Goodfellow PN, Migeon CJ, Smith KD, Berkovitz GD. The role of the sex-determining region Y gene in the etiology of 46,XX maleness. J Clin Endocrinol Metabol 1993;76:690-95.
91. Vorona E, Zitzmann M, Gromoll J, Schuring AN, Nieschlag E. Clinical, endocrinological, and epigenetic features of the 46, XX male syndrome, compared with 47, XXY Klinefelter patients. J Clin Endocrinol Metab 2007;92:3458-65.
92. Ferguson-Smith MA, Cooke A, Affara NA, Boyd E, Tolmie JE. Genotype-phenotype correlations in XX males and their bearing on current theories of sex determination. Hum Genetics 1990;84:198-202.
93. Gotz MJ, Johnstone EC, Ratcliffe SG. Criminality and antisocial behaviour in unselected men with sex chromosome abnormalities. Psychol Med 1999;29:953-62.
94. Witkin HA, Mednick SA, Schulsinger F, Bakkestrom E, Christiansen KO, Goodenough DR, Hirschhorn K, Lundsteen C, Owen DR, Philip J, Rubin DB, Stocking M. Criminality in XYY and XXY men. Science 1976;193:547-55.
95. De Braekeleer M, Dao TM. Cytogenetic studies in male infertility: a review. Hum Reprod 1991;6:245-50
96. Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Vidal F, Egozcue J. Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. Hum Reprod Update 2000;6:93-105.

97. Egozcue J, Blanco J, Anton E, Egozcue S, Sarrate Z, Vidal F. Genetic analysis of sperm and implications of severe male infertility-a review. Placenta 2003;24 Suppl 2:S62-65.
98. Martin RH, Rademaker AW, Greene C, Ko E, Hoang T, Barclay L, Chernos J. A comparison of the frequency of sperm chromosome abnormalities in men with mild, moderate, and severe oligozoo-spermia. Biol Reprod 2003;69:535-9.
99. Martin RH, Greene C, Rademaker AW, Ko E, Chernos J. Analysis of aneuploidy in spermatozoa from testicular biopsies from men with nonobstructive azoospermia. J Androl 2003;24:100-3.
100. Gardner RJM, Sutherland GR. Chromosome abnormalities and genetic counselling. New York: Oxford University Press, 1996.
101. Van Assche E, Bonduelle M, Tournaye H, Joris H, Verheyen G, Devroey P, van Steirteghem A, Liebaers I. Cytogenetics of infertile men. Hum Reprod 1996;11(Suppl 4):1-26.
102. Montag M, van der Ven K, Ved S, Schmutzler A, Prietl G, Krebs D, Peschka B, Schwanitz G, Albers P, Haidl G, van der Ven H. Success of intracytoplasmic sperm injection in couples with male and/or female chromosome aberrations. Hum Reprod 1997;12:2635-40.
103. Meschede D, Horst J. Genetic counselling for infertile male patients. In J Androl 1997;20(Suppl. 3):20-30.
104. Johnson MD. Genetic risks of intracytoplasmic sperm injection in the treatment of male infertility: recommendations for genetic counseling and screening. Fertil Steril 1998;70:397-411.
105. Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, Liebaers I. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod 2002;17:2600-14.
106. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T, Chinwalla A, Delehaunty A, Delehaunty K, Du H, Fewell G, Fulton L, Fulton R, Graves T, Hou SF, Latrielle P, Leonard S, Mardis E, Maupin R, McPherson J, Miner T, Nash W, Nguyen C, Ozersky P, Pepin K, Rock S, Rohlfing T, Scott K, Schultz B, Strong C, Tin-Wollam A, Yang SP, Waterston RH, Wilson RK, Rozen S, Page DC. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature 2003;423:825-37.
107. Hsu LYF. Phenotype/karyotype correlations of Y chromosome aneuploidy with emphasis on structural aberrations in postnatally diagnosed cases. Am J Med Genet 1994;53:108-40.
108. Tiepolo L, Zuffardi O. Localization of factors controlling spermato-genesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Genet 1976;34:119-24.
109. Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Köhn FM, Schill WB, Farah S, Ramos C, Hartmann M, Hartschuh W, Meschede D, Behre HM, Castel A, Nieschlag E, Weidner W, Grone HJ, Jung A, Engel W, Haidl G. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. Hum Mol Genet 1996;5:933-43.
110. Simoni M, Gromoll J, Dworniczak B, Rolf C, Abshagen K, Kamischke A, Carani C, Meschede D, Horst J, Behre HM, Nieschlag E. Screening for deletions of the Y chromosome involving the DAZ (Deleted in Azoospermia) gene in azoospermia and severe oligozoospermia. Fertil Steril 1997;67:542-47.
111. Foresta C, Moro E, Ferlin A. Y chromosome microdeletions and alterations of spermatogenesis. Endocr Rev 2001;22:226-39.
112. Maurer B, Gromoll J, Simoni M, Nieschlag E. Prevalence of Y chromosome microdeletions in infertile men who consulted a tertiary care medical centre: the Muenster experience. Andrologia 2001;33:27-33.
113. Simoni M, Tüttelmann F, Gromoll J, Nieschlag E. Clinical consequences of microdeletions of the Y chromosome: the extended Münster experience. RBMOnline 2008;16:289-303.
114. Noordam MJ, Repping S. The human Y chromosome: a masculine chromosome. Curr Opin Genet Dev 2006;16:225-32.
115. Krausz C, Forti G, McElreavey K. The Y chromosome and male fertility and infertility. Int J Androl 2003;26:70-5.
116. Kamp C, Huellen K, Fernandes S, Sousa M, Schlegel PN, Mielnik A, Kleiman S, Yavetz H, Krause W, Kupker W, Johannisson R, Schulze W, Weidner W, Barros A, Vogt PH. High deletion frequency of the complete AZFa sequence in men with Sertoli-cell-only syndrome. Mol Hum Reprod 2001;7:987-94.
117. Kent-First M, Muallem A, Shultz J, Pryor J, Roberts K, Nolten W, Meisner L, Chandley A, Gouchy G, Jorgensen L, Havighurst T, Grosch J. Defining regions of the Y-chromosome responsible for male infertility and identification of a fourth AZF region (AZFd) by Y-chromosome microdeletion detection. Mol Reprod Dev 1999;53: 27-41.
118. Simoni M, Bakker E, Krausz C. EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. State of the art 2004. Int J Androl 2004;27:240-9.
119. Kamischke A, Gromoll J, Simoni M, Behre HM, Nieschlag E. Transmission of a Y chromosomal deletion involving the deleted in azoospermia (DAZ) and chromodomain (CDY1) genes from father to son through intracytoplasmic sperm injection. Hum Reprod 1999;14:2320-22.
120. Comhaire FH, de Kretser DM, Farley TM, et al. Towards more objectivity in diagnosis and management of male infertility. Int J Androl Suppl 1987;7:1-53.
121. Baker HWG, Burger HG, de Kretser DM, et al. Relative incidence of etiologic disorders in male infertility. In Santen RJ, Swerdloff, R. S. (eds): Male reproductive dysfunction: Diagnosis and management of

hypogonadism, infertility and impotence. New York, Marcel Dekker Inc,1986;341-72.

122. Baker HWG, Burger HG, de Kretser DM, et al. Testicular vein ligation and fertility in men with varicoceles. BMJ 1985;291:1678-80.
123. Hargreave TB. Varicocele: Overview and commentary on the results of the world health organisation varicocele trial. In Waites GMH, Frick J, Baker HWG (eds): Current advances in andrology, Bologna, Monduzzi Editore,1997;31-44.
124. Evers JL, Collins JA. Surgery or embolisation for varicocele in subfertile men. Cochrane Database Syst Rev CD000479, 2004.
125. Ficarra V, Cerruto MA, Liguori G, et al. Treatment of varicocele in subfertile men: The Cochrane review—a contrary opinion. Eur Urol 2006;49:258-63.
126. WHO. The influence of varicocele on parameters of fertility in a large group of men presenting to infertility clinics. World Health Organization. Fertil Steril 1992;57:1289-93.
127. Heshmat S, Lo KC. Evaluation and treatment of ejaculatory duct obstruction in infertile men. Can J Urol 2006;13 Suppl 1:18-21.
128. Hirsch IH, Sedor J, Kulp D, et al. Objective assessment of spermatogenesis in men with functional and anatomic obstruction of the genital tract. Int J Androl 1994;17:29-34.
129. Handelsman DJ, Conway AJ, Boylan LM, et al. Young's syndrome. Obstructive azoospermia and chronic sinopulmonary infections. N Engl J Med 1984;310:3-9.
130. Hendry WF, A'Hern RP, Cole PJ. Was Young's syndrome caused by exposure to mercury in childhood? BMJ 1993;307:1579-82.
131. Gondos B. Ultrastructure of developing and malignant germ cells. Eur Urol 1993;23:68-75.
132. Jørgensen N, Rajpert-De Meyts E, Græm N, Müller J, Giwercman A, Skakkebaek NE. Expression of immunohistochemical markers for testicular carcinoma in situ by normal human germ cells. Lab Invest 1995;72:223-31.
133. Rajpert-De Meyts E, Bartkova J, Samson M., Hoei-Hansen CE, Frydelund-Larsen L, Bartek J, Skakkebæk NE. The emerging phenotype of the testicular carcinoma in situ cell. APMIS 2003;111:267-79.
134. Rajpert-De Meyts E, Jørgensen N, Müller J, Skakkebaek NE. Prolonged expression of the c-kit receptor in germ cells of intersex fetal testes. J Pathol 1996;178:166-9.
135. Bartkova J, Rajpert-De Meyts E, Skakkebæk NE, Bartek J. D-type cyclins in adult human testis and testicular cancer: Relation to cell type, proliferation, differentiation, and malignancy. J Pathol 1999;187:573-81.
136. Bartkova J, Thulberg M, Rajpert-De Meyts E, Skakkebæk NE, Bartek J. Lack of p19INK4d in human testicular germ-cell tumours contrasts with high expression during normal spermatogenesis. Oncogene 2000;19:4146-49.
137. Bartkova J, Rajpert-De Meyts E, Skakkebaek NE, Lukas J, Bartek J. Deregulation of the G1/S-phase control in human testicular germ cell tumours. APMIS 2003;111:252-56.
138. Chaganti RSK, Houldsworth J. Genetics and biology of adult human male germ cell tumors. Cancer Res 2000;60:1475-82.
139. Rajpert-De Meyts E, Skakkebaek NE. The possible role of sex hormones in the development of testicular cancer. Eur Urol 1993;23:54-61.
140. Rivas A, Fisher JS, McKinnell C, Atanassova N, Sharpe RM. Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. Endocrinology 2002;143: 4797-808.
141. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Müller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996;104(Suppl 4):741-803.
142. Skakkebaek NE, Rajpert-De Meyts E, Jørgensen N, Carlsen E, Petersen PM, Giwercman A, Andersen A-G, Jensen TK, Andersson A-M, Müller J. Germ cell cancer and disorders of spermatogenesis: An environmental connection? APMIS 1998;106:3-12.
143. Sharpe RM, Skakkebaek NE. Are oestrogens involved in in falling sperm counts and disorders of the male reproductive tract? Lancet 1993;341:1392-95.
144. Comhaire FH, de Kretser DM, Farley TM, et al. Towards more objectivity in diagnosis and management of male infertility. Int J Androl Suppl 1987;7:1-53.
145. Baker HWG, Burger HG, de Kretser DM, et al. Relative incidence of etiologic disorders in male infertility. In Santen RJ, Swerdloff, R. S. (eds): Male reproductive dysfunction: Diagnosis and management of hypogonadism, infertility and impotence. New York, Marcel Dekker Inc,1986;341-72.
146. Brown DJ, Hill ST, Baker HWG. Male fertility and sexual function after spinal cord injury. Prog Brain Res 2006;152:427-39.
147. Kamischke A, Nieschlag E. Update on medical treatment of ejaculatory disorders. Int J Androl 2002;25:333-44.
148. Colpi G et al. EAU guidelines on ejaculatory dysfunction. Eur Urol 2004;46:555-8.
149. Waldinger MD. The neurobiological approach to premature ejaculation. Jour of Urol, 2002;168:2359-67.
150. Jannini EA et al. Update on pathophysiology of premature ejaculation: the basis for new pharmacological treatments. EAU- EBU Update series 2006;4:141-9.
151. Jannini EA et al. Thyroid hormone and male gonadal function. Endocrinol Rev 1995;16:443-59.

152. Arimura A, Kastin AJ, Gonzales-Barcena D, Siller J, Weaver RE, Schally AV. Disappearance of LH-releasing hormone in man as determined by radioimmunoassay 1974;3:421-5.
153. Pimstone B, Epstein S, Hamilton SM, LeRoith D, Hendricks S. Metabolic clearance and plasma half-disappearance time of exogenous gonadotropin-releasing hormone in normal subjects and in patients with liver disease and chronic renal failure. Journal of Clinical Endocrinology and Metabolism 1977;44:356-60.
154. Santoro N, Filicori M, Crowley Jr WF. Hypogonadotropic disorders in men and women: diagnosis and therapy with pulsatile gonadotropin-releasing hormone. Endocrine Reviews 1986;7:11-23.
155. Spratt DI, Carr DB, Merriam GR, Scully RE, Rao PN, Crowley Jr WF. The spectrum of abnormal patterns of gonadotropin-releasing hormone secretion in men with idiopathic hypogonadotropic hypogonadism: clinical and laboratory correlations. Journal of Clinical Endocrinology and Metabolism 1987;64:283-91.
156. Beitins IZ, Dufau ML, O'Loughlin K, Catt KJ, McArthur JW. Analysis of biological and immunological activities in the two pools of LH released during constant infusion of luteinizing hormone-releasing hormone (LHRH) in men. Journal of Clinical Endocrinology and Metabolism 1977;45:605-8.
157. Kallmann FJ, Schoenfeld WA. The genetic aspects of primary eunuchoidism. American Journal of Mental Deficiency 1944;158:203-36.
158. Bick D, Franco B, Sherins RJ, et al. Brief report: intragenic deletion of the KALIG-1 gene in Kallmann's syndrome. New England Journal of Medicine 1992;326:1752-5.
159. Edelman GM, Crossin KL. Cell adhesion molecules: implications for a molecular histology 1991;60:155-90.
160. Hardelin JP, Levilliers J, Blanchard S, et al. Heterogeneity in the mutations responsible for X chromosome- linked Kallmann syndrome. Human Molecular Genetics 1993;2:373-7.
161. Georgopoulos NA, Pralong FP, Seidman CE, Seidman JG, Crowley Jr WF, Vallejo M. Genetic heterogeneity evidenced by low incidence of KAL-1 gene mutations in sporadic cases of gonadotropin-releasing hormone deficiency. Journal of Clinical Endocrinology and Metabolism 1997;82:213-7.
162. Pitteloud N, Hayes FJ, Boeppk PA, DeCruz S, Seminere SB, McLaughlin DT, Crowley WF, Jr. The role of prior pubertal development, biochemical markers of testicular maturation, and genetics in deciding the phenotype heterogeneity of idiopathic hypogonadotrpic hypogonadism. J Clin Endocrinol Metab 2002;87:152-60.
163. Zanaria E, Muscatelli F, Bardoni B, et al. An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature 1994;372:635-41.
164. Caron P, Imbeaud S, Bennet A, Plantavid M, Camerino G, Rochiccioli P. Combined hypothalamic-pituitary-gonadal defect in a hypogonadic man with a novel mutation in the DAX-1 gene. J Clin Endocrinol Metab 1999;84:3563-9.
165. Seminara SB, Achermann JC, Genel M, Jameson JL, Crowley WF, Jr. X-linked adrenal hypoplasia congenita: a mutation in DAX1 expands the phenotypic spectrum in males and females. J Clin Endocrinol Metab 1999;84: 4501-9.
166. Tabarin A, Achermann JC, Recan D, et al. A novel mutation in DAX1 causes delayed-onset adrenal insufficiency and incomplete hypogonadotropic hypogonadism. J Clin Invest 2000;105:321-8.
167. Layman LC, Wilson JT, Huey LO, Lanclos LD, Plouffe Jr L, McDonough PG. Gonadotropin-releasing hormone, follicle-stimulating hormone beta, luteinizing hormone beta gene structure in idiopathic hypogonadotropic hypogonadism. Fertility and Sterility 1992;57:42-49.
168. Weiss J, Crowley Jr WF, Jameson JL. Normal structure of the gonadotropin-releasing hormone (GnRH) gene in patients with GnRH deficiency and idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab 1989;69:299-303.
169. de Roux N, Young J, Brailly-Tabard S, Misrahi M, Milgrom E, Schaison G. The same molecular defects of the gonadotropin-releasing hormone receptor determine a variable degree of hypogonadism in affected kindred. Journal of Clinical Endocrinology and Metabolism 1999;84:567-72.
170. Caron P, Chauvin S, Christin-Maitre S, et al. Resistance of hypogonadic patients with mutated GnRH receptor genes to pulsatile GnRH administration. Journal of Clinical Endocrinology and Metabolism 1999;84:990-6.
171. Layman LC, Peak DB, Xie J, Sohn SH, Reindollar RH, Gray MR. Mutation analysis of the gonadotropin-releasing hormone receptor gene in idiopathic hypogonadotropic hypogonadism. Fertility and Sterility 1997;68:1079-85.
172. Pralong FP, Gomez F, Castillo E, et al. Complete hypogonadotropic hypogonadism associated with a novel inactivating mutation of the gonadotropin-releasing hormone receptor. Journal of Clinical Endocrinology and Metabolism 1999;84:3811-6.
173. Seminara SB, Beranova M, Oliveira LMB, Martin KA, Crowley Jr WF, Hall JE. Successful use of pulsatile gonadotropin-releasing hormone (GnRH) for ovulation induction and pregnancy in a patient with GnRH receptor mutations. Journal of Clinical Endocrinology and Metabolism 2000;85:3027-35.
174. Dode C, Levilliers J, Dupont JM, et al. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nature Genet 2003;33:463-5.
175. de Roux, N.; Genin, E.; Carel, J.-C et al. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Nat Acad Sci 2003;100:10972-6.
176. Seminara SB.; Messager, S.; Chatzidaki, E. E. et al. The GPR54 gene as a regulator of puberty. New Eng. J Med 2003;349:1614-27.
177. Weiss J, Axelrod L, Whitcomb RW, Harris PE, Crowley Jr WF, Jameson JL. Hypogonadism caused by a single

amino acid substitution in the beta subunit of luteinizing hormone. N Engl J Med 1992;326:179-83.
178. Lindstedt G, Nystrom E, Matthews C, Ernest I, Janson PO, Chatterjee K. Follitropin (FSH) deficiency in an infertile male due to FSH beta gene mutation. A syndrome of normal puberty and virilization but underdeveloped testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations. Clin Chem Lab Med 1998;36:663-5.
179. Phillip M, Arbelle JE, Segev Y, Parvari R. Male hypogonadism due to a mutation in the gene for the beta subunit of follicle stimulating hormone. New Engl J Med 1998;338:1729-32.
180. Parks JS, Brown MR, Hurley DL, Phelps CJ, Wajnrajch MP. Heritable disorders of pituitary development. J Clin Endocrinol Metab 1999;84:4362-70.
181. Dorr H, Bohring C, Krause W. Are antisperm antibodies indeed sperm-specific? Andrologia 2005;37:185-7.
182. Troedsson MH, Desvousges A, Alghamdi AS *et al.* Components in seminal plasma regulating sperm transport and elimination. Anim Reprod Sci 2005;89:171-86.
183. Rooney IA, Oglesby TJ, Atkinson JP. Complement in human reproduction: activation and control. Immunol Res 1993;12,276-94.
184. Sedor J, Callahan HJ, Perussia B, Lattime EC, Hirsch IH. Soluble Fc ã RIII (CD16) and immunoglobulin G levels in seminal plasma of men with immunological infertility. J Androl 1993;14:187-93.
185. Ou JP, Zhuang GL. Effect of antisperm antibodies on assisted reproduction. Zhonghua Nan Ke Xue 2003;9:214-7.
186. Munoz MG, Jeremias J, Witkin SS. The 60 kDa heat shock protein in human semen: relationship with antibodies to spermatozoa and Chlamydia trachomatis. Hum Reprod 1996;11:2600-03.
187. Shi J, Yang Z, Wang M et al. Screening of an antigen target for immunocontraceptives from cross-reactive antigens between human sperm and Ureaplasma urealyticum. Infect Immun 2007;75:2004-11.
188. Silber SJ, Grotjan HE. Microscopic vasectomy reversal 30 years later: a summary of 4010 cases by the same surgeon. J Androl 2004;25,845-59.
189. Lee HY, Kim JQ, Kim SI. Sperm auto-immunity associated with vasectomy, vasovasostomy and epididymovasostomy in Korean males. Clin Reprod Fertil 1987;5:347-57.
190. Podzimek S, Prochazkova J, Pribylova L et al. Effect of heavy metals on immune reactions in patients with infertility. Cas Lek Cesk 2003;142: 285-8.
191. Podzimek S, Prochazkova J, Bultasova L et al. Sensitization to inorganic mercury could be a risk factor for infertility. Neuro Endocrinol. Lett 2005;26:277-82.
192. Goto A. Histological and immunohistochemical studies on the damage of contralateral testis following the unilateral testicular torsion in Wistar rats. Nippon Hinyokika Gakkai Zasshi 1991;82:726-33.
193. Bahraminejad R, Kadanali S, Erten O, Bahar H. Reproductive failure and antisperm-antibody production among prostitutes. Acta Obstet. Gynecol. Scand 1991; 70:483-5.
194. Bohring C, Klepper L, Krause W. Localization of binding sites of naturally occurring antisperm antibodies on human spermatozoa by immunofluorescence. Andrologia 2004;36:286-90.
195. D'Cruz OJ, Haas GG Jr. Lack of complement activation in the seminal plasma of men with antisperm antibodies associated in vivo on their sperm. Am. J. Reprod. Immunol 1990;24:51-57.
196. D'Cruz OJ, Toth CA, Haas GG Jr. Recombinant soluble human complement receptor type 1 inhibits antisperm antibody- and neutrophil-mediated injury to human sperm. Biol. Reprod 1996;54:1217-28.
197. D'Cruz OJ, Haas GG Jr, Wang BL, DeBault LE. Activation of human complement by IgG antisperm antibody and the demonstration of C3 and C5b-9-mediated immune injury to human sperm. J. Immunol 1991;146:611-20.
198. D'Cruz OJ, Haas GG Jr. b2-integrin (CD11b/CD18) is the primary adhesive glycoprotein complex involved in neutrophil-mediated immune injury to human sperm. Biol. Reprod 1995;53:1118-30.
199. Eggert-Kruse W, Bockem-Hellwig S, Doll A, Rohr G, Tilgen W, Runnebaum B. Antisperm antibodies in cervical mucus in an unselected subfertile population. Hum. Reprod 1993;8:1025-31.
200. Chantler E, Sharma R, Sharman D. Changes in cervical mucus that prevent penetration by spermatozoa. Symp. Soc. Exp. Biol 1989;43:325-36.
201. Liu DY, Martic M, Clarke GN et al. An anti-actin monoclonal antibody inhibits the zona pellucida-induced acrosome reaction and hyperactivated motility of human sperm. Mol. Hum. Reprod 2002;8:37-47.
202. Santhanam R, Naz RK. Novel human testis-specific cDNA: Molecular cloning, expression and immunobiological effects of the recombinant protein. Mol. Reprod. Dev 2001;60:1-12.
203. Munoz MG, Witkin SS. Autoimmunity to spermatozoa, asymptomatic Chlamydia trachomatis genital tract infection and γδT lymphocytes in seminal fluid from the male partners of couples with unexplained infertility. Hum. Reprod 1995;10:1070-4.
204. Hisaeda H, Nagasawa H, Maeda K et al. γδT cells play an important role in hsp65 expression and in acquiring protective immune responses against infection with Toxoplasma gondu. J. Immunol 1995;154:244-51.
205. Gil T, Castilla JA, Hortas ML et al. Increase of large granular lymphocytes in human ejaculate containing antisperm antibodies. Hum. Reprod 1998;13:296-301.
206. Hill JA, Cohen J, Anderson DJ. The effects of lymphokines and monokines on human sperm fertilizing ability in the zona-free hamster egg penetration test. Am. J. Obstet. Gynecol 1989;160:1154-9.

207. Nakagawa K, Yamano S, Kamada M, Hinokio K, Maegawa M, Aono T. Quality of embryo does not affect the implantation rate of IVF-ET in infertile women with antisperm antibody. Fertil. Steril 1999;72:1055-60.
208. De Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes; and II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J Androl 1992;13:368-386.
209. Gagnon C, Iwasaki A, de Lamirande E, and Kovalski N. Reactive oxygen species and human spermatozoa. Ann N Y Acad Sci 1991;637:436-44
210. Armstrong JS, Bivalacqua TJ, Chamulitrat W, Sikka SC, Hellstrom WJG. A comparison of the NADPH oxidase in human sperm and white blood cells. Int J Androl 2002;25:223-9.
211. Aitken RJ, Paterson M, Fisher H, Buckingham DW, Van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci 1995;108:2017-25.
212. Hellstrom WJG, Bell M, Wang R, Sikka SC. Effect of sodium nitroprusside on sperm motility, viability and lipid peroxidation. Fertil. Steril 1994;61:1117-22.
213. Ernster L. Lipid peroxidation in biological membranes: mechanisms and implications. In: Yagi K, ed. Active oxygen, lipid peroxides and antioxidants. Boca Raton, FL: CRC Press; 1993:1-38.
214. Kodama H, Kuribayashi Y, Gagnon C. Effect of lipid peroxidation on fertilization. J Androl 1996;17:151-7.
215. Jones R, Mann T, Sherins RJ. Peroxidative breakdown of phospholipids in human spermatozoa: spermicidal effects of fatty acid peroxides and protective action of seminal plasma. Fertil Steril 1979;31:531-7.
216. Geva E, Lessing JB, Lerner-Geva L, Amit A. Free radicals, antioxidants and human spermatozoa: clinical implications. Hum Reprod 1998;13:1422-4.
217. Martin-Du Pan RC, Sakkas D. Are antioxidants useful in the treatment of male infertility? Hum Reprod 1998; 13:2984-5.
218. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999;14:2801-7.
219. Donnelly E, McClure N, Lewis SEM. Antioxidant supplementation in vitro does not improve human sperm motility. Fertil Steril 1999;72:484-95.
220. Agarwal A. Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am 2002;29:817-27.
221. Alvarez JG, Storey BT. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res 1989;23:77-90.
222. Bell M, Wang R, Hellstrom WJG, Sikka SC. Effect of cryopreservation protocol on sperm membrane lipid peroxidation and recovery of motile human sperm. J Androl 1993;14:472-8.
223. Irvine DS. Glutathione as a treatment for male infertility. Rev Reprod 1996;1:6-12.
224. Leclerc P, de Lamirande E, Gagnon C. Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic Biol Med 1997;22:643-56.
225. Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJG, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Radic Biol Med 1999;26:869-80.
226. Jain SK, Palmer M. The effect of oxygen radical metabolites and vitamin E on glycosylation of proteins. Free Radic Biol Med 1997;22:593-6.
227. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:1-15.
228. Aaberg RA, Sikka SC, Sauer MV, Rajfer J. Effect of extracellular ionized calcium, diltiazem and cAMP on human spermatozoa motility. J Urol 1989;14:1221-4.
229. Sun J-G, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 1997; 56:602-7.
230. Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts IUI outcome: a prospective cohort study. Hum Reprod 2002;12:3122-8.
231. Sakkas D, Moffatt O, Manicardi G, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and possible involvement of apoptosis. Biol Reprod 2002;66:1061-7.
232. Kao SH, Chao HT, Wei YH. Multiple deletions of mtDNA are associated with decline of motility and fertility of human spermatozoa. Mol Hum Reprod 1998;4:657-66.
233. Evenson DP, Larson KJ, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002;23:25-43.
234. Sakkas D, Moffatt O, Manicardi G, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and possible involvement of apoptosis. Biol Reprod 2002;66:1061-7.

CHAPTER 5

Evaluating the Infertile Male

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ABSTRACT

Male infertility solely constitutes 20% of the infertile population. Moreover, some contemporary reports suggest that the number of subfertile males is on the increase. Advances in various assisted reproduction techniques allow many subfertile and infertile men to father a child. Despite these developments, the proper evaluation of the infertile male remains crucial for the diagnosis of potentially correctable and uncorrectable causes of male factor infertility for ultimate management in a cost effective manner.

INTRODUCTION

Infertility is the inability to conceive after one year of unprotected intercourse. Almost 15% of couples fall into this subset of the general population. The male factor contributes exclusively to 20% of the problem, while 30-40% of infertility cases are due to male and female factors combined.[1] Despite the definition of infertility, in certain circumstances, the evaluation of an infertile couple may be critical before passing the defined period of one year. Evident infertility risk factors such as previous surgeries on the male reproductive tract, large varicoceles or advanced female partner age especially over 35 years may warrant prompt evaluation of the infertile couple. Premarital fertility assessment may also be another indication for early evaluation.[2]

Male factor evaluation must be conducted in a rapid, systematic, cost effective fashion, and most importantly, synchronized with the evaluation of the female partner. The main goal of this evaluation is to ascertain the potentially correctable causes of male infertility, as well as the uncorrectable causes. Cases where infertility is incurable will eventually require either assisted reproduction or alternative options such as adoption.[2] Moreover, 1 to 10% of cases of male infertility may be due to a life-threatening, yet curable medical condition which further underscores the importance of a thorough clinical and lab evaluation of every infertile male.[3]

The cornerstones of a male fertility evaluation include initial screening by taking a thorough history, conducting a meticulous physical examination, and testing semen on two different occasions two to four weeks apart, followed by a cost-effective battery of laboratory and radiological investigations to confirm the diagnosis (Figure 5.1). This approach will aid the infertility specialist in planning treatment and provide a practical case-specific prognosis for proper patient counseling.

HISTORY

A thorough history of an infertile male should include his complete medical, surgical and sexual background with special emphasis on factors related to male infertility. The infertility specialist should be aware of the female partner's condition while evaluating the male partner. Many causes of male infertility may be identified through patient history. History taking should address seven major areas, starting with a sexual history that includes frequency and timing of sexual intercourse in relation to the female partner's ovulatory cycle. History of sexually transmitted diseases is also important due to their detrimental effects on the reproductive organs of both partners. Pregnancy history should be the second line of inquiry. Whether this is a case of primary or

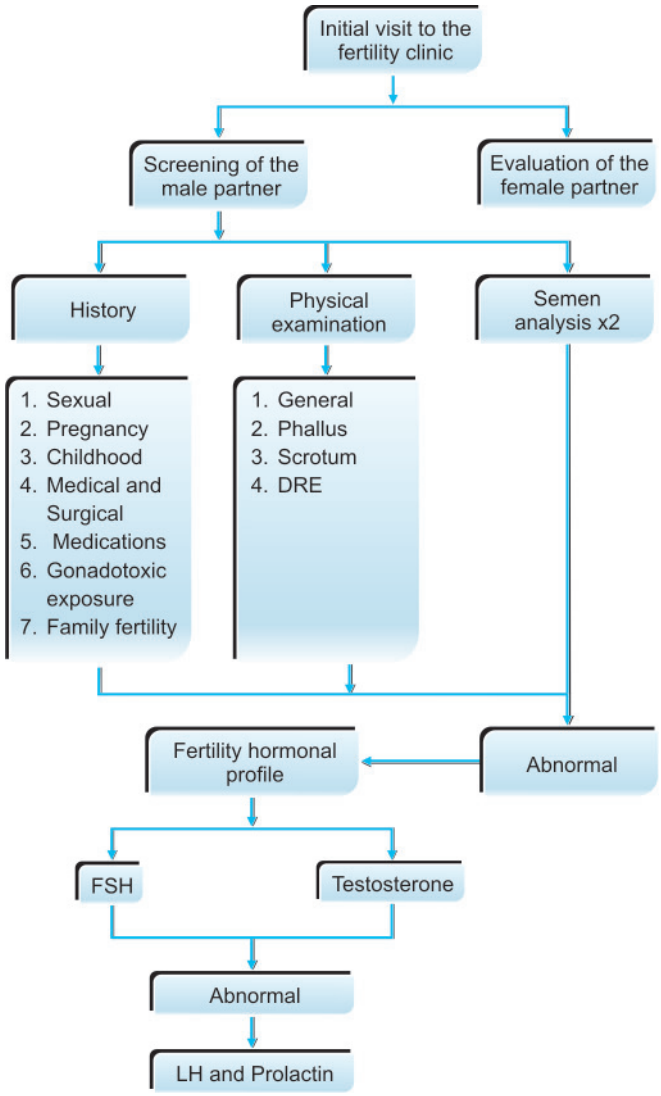


Figure 5.1: Algorithm for the primary evaluation of the infertile male

secondary infertility and the duration of infertility also have a prognostic value. A history of childhood diseases such as viral orchitis or cryptorchidism may delineate testicular causes of infertility. Other causes of male infertility may be identified through the medical and surgical history. Diseases such as diabetes mellitus and multiple sclerosis may cause erectile and ejaculatory dysfunction. Recurrent respiratory tract infections can be a result of various syndromes associated with infertility such as Young's syndrome and Kartagener's syndrome. A recent high fever may cause temporary impairment of spermatogenesis. Previous pelvic or inguinal surgery, or trauma may be a direct cause of infertility. Current medications should be carefully addressed as some drugs may be gonadotoxic, while some hormones such as steroids may be contraceptive (Table 5.1).

History of exposure to gonadotoxins is another critical issue to be discussed, including inquiry about the patient's occupation. Smoking, cytotoxic therapies, drugs of abuse and heat are all well-known for their adverse effects on spermatogenesis and fecundity. Finally, investigating the family reproductive history may help outline certain hereditary causes of infertility such as cystic fibrosis or androgen receptor deficiency.

Physical Examination

The physical examination begins as soon as the patient enters through the door of the infertility specialist's office. The patient's overall body habitus may be the primary marker of endocrinal homeostasis within the body. Secondary sexual characters such as body hair distribution should be assessed. Eunuchoid proportions and gynecomastia, anosmia or situs invertus are all characteristic signs of different syndromes associated with male infertility such as Klinefelter, Kallmann or Kartagener's syndrome, respectively. Obesity may cause infertility systemically by disturbing hormonal levels in the body or locally by elevating the temperature of the testes, thus hindering normal spermatogenesis.

A meticulous local examination of the genitalia follows the general examination. The location of the urethral meatus and the normal alignment of the penis are important factors for the proper deposition of the ejaculate within the female reproductive tract. The penile shaft is examined for hypospadias, epispadias or

Table 5.1: Potentially gonadotoxic drugs[4]

- Alcohol
- Alkylating agents (e.g. cyclophosphamide)
- Allopurinol
- Antipsychotics
- Arsenic
- Aspirin (large doses)
- Caffeine
- Calcium channel blockers
- Cimetidine
- Cocaine
- Colchicine
- Dibromochloropropane (pesticides)
- Diethylstilbestrol (DES)
- Lead
- Lithium
- Marijuana
- Medroxyprogesterone
- Monoamine oxidase inhibitors
- Nicotine
- Nitrofurantoin
- Phenytoin
- Spironolactone
- Sulfasalazine
- Testosterone
- Tricyclic antidepressants
- Valproic acid

abnormal curvature during erection. Seminiferous tubules, which are responsible for spermatogenesis, constitute around 80% of the testicular volume, making the size and consistency of both testes important predictors of testicular function. The normal testis ranges from 15-20 mL in volume, feels turgid in consistency and must be devoid of any abnormal swellings on palpation. The epididymis must be palpated to show a head, body and tail. Any cystic dilation or indurations may denote the possibility of an obstruction. Spermatoceles and epididymal cysts, although common findings, do not indicate obstruction.[5] Palpation of the spermatic cord follows to confirm the presence of both vas deferens, excluding unilateral or bilateral absence of the vas deferens and identifying any other pathology within the deferential ducts that could be a cause of obstruction, such as areas of induration or nodules. Examination of the cord contents also rules out the presence of a varicocele, which is basically a diagnosis of palpation rather than imaging. The patient must be examined in a warm room while standing. Reexamining the patient in the lying position may be useful in discovering rare cases of intra-abdominal masses that obscure venous return within varicoceles. The presence of a palpable varicocele stands as one of the indications for varicocele ligation according to the current American Society for Reproductive Medicine guidelines.[6]

Finally, a digital rectal examination is performed to identify any infections or masses within the prostate or dilation of the seminal vesicles as in cases of obstruction.

Ancillary Examination Methods

Scrotal Doppler Ultrasound

Indications for scrotal Doppler ultrasound in the infertility clinic include either the presence of a risk factor for germ cell tumors (e.g. history of cryptorchidism or previous germ cell tumor), unsuccessful physical examination of the testes (e.g. inguinal testes), prior testicular surgery or to confirm the presence of a varicocele.[7] Nevertheless, some recommend performing a Doppler ultrasound on every patient with unexplained infertility or idiopathic abnormal semen analysis to rule out pathologic conditions such as testicular malignancy that can be missed on physical examination[8-10] (Table 5.2).

Table 5.2: Findings on scrotal color Doppler ultrasonography in 1,372 infertile men[10]

Findings	*No. cases (%)*	
Varicoceles	407	(29.7%)
Epididymal cysts	104	(7.6%)
Hydroceles	44	(3.2%)
Testicular microlithiasis	12	(0.9%)
Testicular cysts	9	(0.7%)
Testicular tumors	7	(0.5%)

Doppler ultrasound allows evaluation of the hemodynamic repercussion of a varicocele by studying the spectral display, color and response to Valsalva. It also provides an exact measure of testicular volume and detects the presence of dystrophic changes or lesions in the testis in addition to anomalies in the epididymis and vas deferens, such as cystic dilatations. Moreover, Doppler ultrasound is the standard radiological tool for the diagnosis of undescended testicles.[8]

Varicoceles are found in 40% of infertile men[11] and 70% of men with secondary infertility.[12] Doppler ultrasound may be used to detect subclinical varicoceles in infertile men but not routinely in cases of clinically evident varicoceles.[13] A varicocele is diagnosed by measuring the internal spermatic vein diameter, with the ultrasound performed while the patient is at rest in the supine position. Radiological criteria for the diagnosis of a varicocele are a spermatic vein exceeding 3 mm in diameter and/or a rise in vein diameter by 1 mm during the Valsalva maneuver[14,15] with an estimated cutoff point for venous diameter above or equal to 3.6 mm for a clinical varicocele and 2.7 mm for a subclinical varicocele. Nevertheless, this test was documented to have an overall accuracy reaching only 63%.[16] Measuring a venous diameter of 3 mm may sometimes be inadequate for diagnosing or ruling out a varicocele,[17] which confirms varicoceles to be a diagnosis of physical examination. To date the clinical relevance of subclinical varicoceles remains controversial.

Epididymal cysts have an uncertain clinical significance and are not specific for epididymal obstruction.[8] Although the natural history of *testicular microlithiasis* is not well defined, it has been associated with cryptorchidism, varicocele, epididymal cysts, infertility, carcinoma in-situ (CIS) and testicular tumors.[19] *Testicular cysts* require careful follow up owing to the possibility of them incorporating a teratoma.[18] Several studies have

suggested that male infertility and *testicular* tumors may share common etiological factors. Infertile men were observed to be 3 times more likely to develop testicular cancer compared to those without infertility.[20] Moreover, the presence of an abnormal semen analysis was associated with a 20-fold increase in this risk compared to the general population,[21] with seminoma being the most frequently seen tumor.[20]

Transrectal Ultrasound (TRUS)

High resolution TRUS is an important diagnostic tool when ejaculatory duct obstruction (EDO), or seminal vesicle agenesis or hypoplasia is suspected. Classically, these patients have azoospermia with low seminal volume and reduced seminal pH. TRUS findings in suspected EDO include midline cysts, dilated seminal vesicles or ejaculatory ducts, or hyperechoic regions suggestive of calcifications.[22,23] Müllerian duct cysts may be present in 11% of infertile men and may be a cause of EDO.[24] Although not pathognomonic, seminal vesicles larger than 15 mm in transverse diameter suggest duct obstruction. TRUS-guided transrectal seminal vesiculography is performed under local anesthesia using a 22G needle advanced into the seminal vesicles. A positive fluid aspirate confirms the diagnosis of EDO. Contrast media also may be injected to identify the site of obstruction.[24,25]

In the past, vasography was the gold standard for diagnosis of seminal duct obstruction. However, TRUS is much less invasive and can elaborately demonstrate the anatomic relationships of the prostate, seminal vesicles and ejaculatory ducts with exquisite detail. TRUS also allows the display of the ampullary part of the contralateral vas deferens and the seminal vesicle. It is also important in cases of unilateral vasal agenesis where contralateral segmental atresia of the vas deferens or seminal vesicle is associated resulting in azoospermia.[23,25]

Occasionally, a complimentary abdominal ultrasound in men with vasal agenesis may detect renal abnormalities in over 25% of men with unilateral disease and over 10% of men with congenital bilateral absence of the vas dererens (CBAVD).[26]

Thermography

Elevated testicular temperature has an important role in the pathophysiology of male infertility. There are three forms of thermography: liquid contact thermography (LCT), infrared thermography (IRT) and microwave thermography (MWT). Some authors advocate the use of IRT for long term follow up of varicoceles in the pediatric population.[27] Despite the role of scrotal thermography in the evaluation of testicular function associated with varicoceles in pre-pubertal patients that cannot conduct a semen analysis, it has not been used as a screening test in infertile men because of its very low specificity for varicocele detection.[28]

Vasography

Vasography is a slightly more invasive radiological diagnostic technique. It involves the injection of a radioopaque dye through the seminal ducts allowing the flow of dye to be traced by X-ray. This test can either confirm patency of the seminal ducts or identify the site of an obstruction as part of planning reconstructive surgery. It can be performed through a percutaneous or an open approach. As a golden rule carrying out a vasography should always be performed on the same day reconstructive surgery is planned due to the risk of vasal scarring and subsequent obstruction caused by the technique itself.[22]

Endocrine Laboratory Diagnosis

Endocrinopathies are found in about 3% of men attending infertility clinics. They are rarely present in patients with sperm counts greater than 10 million per milliliter. Therefore, endocrine screening should be reserved for patients with azoospermia, severe oligospermia or when clinical stigmata raise suspicion, such as in cases of gynecomastia, testicular atrophy, reduced libido or eunuchoid habitus.[5]

Hormonal Screening

Basic hormonal evaluation begins with serum follicular stimulating hormone (FSH) and testosterone (T) obtained in a morning sample.[5,29] Both are essential for normal seminiferous tubule function.[30] Further assays for luteinizing hormone (LH), prolactin, estradiol and thyroid hormones may be obtained depending on the clinical scenario and results of initial studies. Results of hormonal profiling may show normal testicular endocrinal function, primary or secondary hypogonadism, complete testicular failure or hyperprolactinemia[5] (Table 5.3).

In azoospermic men with normal T and FSH levels, obstruction may be excluded by the history and physical

Table 5.3: Endocrinal diagnosis based on serum hormonal testing[5]

Diagnosis	*Testosterone*	*LH*	*FSH*	*Prolactin*
Normal spermatogenesis (obstruction)	Normal	Normal	Normal	Normal
Secondary hypogonadism (e.g. Kallmann syndrome)	↓	↓	↓	Normal
Primary hypogonadism (e.g. Klinefelter syndrome)	↓	↑	↑	Normal
Germ cell failure (e.g. Sertoli cell only)	Normal	Normal	↑	Normal
Prolactin-secreting pituitary tumor	↓	Normal/↓	Normal/↓	↑
Androgen resistance	↑	↑	↑/Normal	Normal

examination in addition to the assessment of seminal composition. Female partners of patients who prove to have congenital anomalies within the vas deferens must undergo cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation analysis before using the sperm of their partners in any treatment procedure. In positive cases, testing the male partner for the CFTR gene mutation becomes mandatory.[22] Azoospermic or severely oligozoospermic men with elevated FSH are suggestive of primary testicular failure. After a thorough physical examination, karyotyping and screening for Yq microdeletions are warranted for these men.[31]

REFERENCES

1. Thonneau P, et al. Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988-1989). Hum Reprod. 1991;6(6):811-6.
2. Report on optimal evaluation of the infertile male. Fertil Steril. 2006;86(5 Suppl):S202-9.
3. Honig SC, Lipshultz LI, Jarow J: Significant medical pathology uncovered by a comprehensive male infertility evaluation. Fertil Steril. 1994;62(5):1028-34.
4. Shefi S, Turek PJ: Definition and current evaluation of subfertile men. Int Braz J Urol. 2006;32(4):385-97.
5. Jarow JP: Diagnostic approach to the infertile male patient. Endocrinol Metab Clin North Am. 2007;36(2):297-311
6. Report on varicocele and infertility. Fertil Steril. 2008;90(5 Suppl):S247-9.
7. Eifler JB Jr., King P, Schlegel PN. Incidental testicular lesions found during infertility evaluation are usually benign and may be managed conservatively. J Urol. 2008;180(1):261-4, discussion 265.
8. Sanchez Guerrero A, Villor Esnal R, Pamplona Casamayor M. Radiological diagnosis: scrotal ultrasound and Doppler ultrasound in the diagnosis of male infertility. Arch Esp Urol 2004;567(9):905-20.
9. Onur MR, et al. Scrotal ultrasonography: should it be used in routine evaluation of infertile men? Andrologia. 2008;40(1):58-61.
10. Pierik FH, et al. Is routine scrotal ultrasound advantageous in infertile men? J Urol, 1999;162(5):1618-20.
11. Jarow JP. Effects of varicocele on male fertility. Hum Repord Update, 2001;7(1):59-64.
12. Witt MA, Lipshultz LI. Varicocele: a progressive or static lesion? Urology, 1993;42(5):541-3.
13. Sfeir RE Wazzan W. Spermatic vein Doppler examination: use and abuse. J Med Liban, 1994;42(2):59-62.
14. Cornud F, et al. Varicocele: strategies in diagnosis and treatment. Eur Radiol, 1999;9(3):536-45.
15. Kondoh N, et al. Significance of subclinical varicocele detected by scrotal sonography in male infertility: a preliminary report. J Urol, 1993;150(4):1158-60.
16. Eskew LA, et al. Ultrasonographic diagnosis of varicoceles. Fertil Steril, 1993;60(4):693-7.
17. Metin A, Bulut O, Temizkan M. Relationship between the left spermatic vein diameter measured by ultrasound and palpated varicocele and Doppler ultrasound findings. Int Urol Nephrol, 1991;23(1):65-8.
18. Heidenreich A, et al. Color Doppler ultrasonography as a routine clinical examination in male infertility. Int J Urol, 1995;153(4):1174-50.
19. Sakamoto H, et al. Color Doppler ultrasonography as a routine clinical examination in male infertility. Int J Urol 2006;13(8):1073-8.
20. Walsh TJ, et al. Increased risk of testicular germ cell cancer among infertile men. Arch Intern Med, 2009;169(4):351-6.
21. Raman JD, Nobert CF, and Goldstein M. Increased incidence of testicular cancer in men presenting with infertility and abnormal seman analysis. J Urol, 2005;174(5):1819-22 discussion 1822.
22. Report on evaluation of the azoospermic male. Fertile Steril, 2006;86(5 Suppl 1):S210-5.

23. Hall S, Oates RD. Unilateral absence of the scrotal vas deferens associated with contralateral mesonephric duct anomalies resulting in infertility: laboratory, physical and radiographic findings, and therapeutic alternatives. J Urol, 1993;150(4):1161-4.
24. Jarow JP, Transrectal ultrasonography of infertile men. Fertil Steril, 1993;60(6):1035-9.
25. Fisch H, Lambert SM, Goluboff ET. Management of ejaculatory duct obstruction: etiology, diagnosis, and treatment. World J Urol, 2006;24(6):604-10.
26. Schlegel PN, Shin D, and Goldstein M. Urogenital anomalies in men with congenital absence of the vas deferens. J Urol, 1996;155(5):1644-8.
27. Saxena AK, Willital GH. Infrared thermography: experience from a decade of pediatric imaging. Eur J Pediatr, 2008;167(7):757-64.
28. Trum JW, et al. The value of palpation, varicoscreen contact thermography and color Doppler ultrasound in the diagnosis of varicocele. Hum Repord, 1996;11(6):1232-5.
29. Sussman EM, Chudonovsky A, and Niederberger CS. Hormonal evaluation of the infertile male: has it evolved? Urol Clin North Am, 2008;35(2):147-55vii.
30. Jarow JP, Endocrine causes of male infertility. Urol Clin North Am, 2003;30(1):83-90.
31. Bhasin S, Approach to the infertile man. J Clin Endocrinol Metab, 2007;92(6):1995-2004.

CHAPTER 6

Management of Male Infertility—Varicocele Repair

John Kefer, Edmund Sabanegh

ABSTRACT

A varicocele is defined as the distention of the veins surrounding the testicle and traveling along the spermatic cord, and is a frequent cause of male infertility. Normally, testicular veins act to cool blood flowing in the testicular artery through the countercurrent exchange mechanism, maintaining the proper temperature for optimal sperm production and motility. Development of a varicocele causes venous congestion and decreased flow, leading to a rise in testicular temperatures which can be harmful for sperm motility and optimum spermatogenesis.

Varicocele repair has been utilized in infertile couples with a high rate of success. Significant innovations in surgical technique for varicocele repair can improve success rates and decrease morbidity.

MANAGEMENT OF MALE INFERTILITY: VARICOCELE REPAIR

Overview

Varicocele is a dilation of the internal spermatic veins and is considered one of the most surgically correctable causes of male infertility. Varicoceles are present in 15% of the normal male population and in up to 40% of patients presenting for fertility evaluation.[1] While the majority occur on the left side due to the acute angle of insertion of the left gonadal vein into the renal vein, they may be bilateral or isolated to the right side. Typically, varicoceles result from incompetent valves within the gonadal veins, and the subsequent venous congestion leads to persistent venous dilation, worsened by gravity in the upright position. Over the past decade, the diagnosis and treatment of varicoceles has advanced significantly. Growing scientific evidence demonstrating the deleterious effects of varicoceles on spermatogenesis has been complemented by significant innovations in surgical technique, leading to more successful outcomes while reducing morbidity.

Preoperative Evaluation

Patients presenting with varicocele require a detailed medical and reproductive history, physical examination and at least two semen analyses. The physical examination is critical in the evaluation and should be performed with the patient in both the recumbent and standing positions. Examination performed with the patient in the upright position usually reveals a mass of dilated, tortuous veins lying posterior to and above the testis, possibly extending up to the external inguinal ring. The venous dilation noted on exam can usually be increased using the Valsalva, or abdominal straining, maneuver.

While most men have a left-sided varicocele, the presence of a unilateral right-sided varicocele raises suspicion for poor drainage of the right testicular or renal vein, possibly due to a retroperitoneal or renal mass producing obstruction of the vena cava. Sudden onset of a varicocele in an older man also may be suggestive of a retroperitoneal mass leading to inadequate drainage of the testicular veins. Bilateral varicoceles are also becoming more frequently diagnosed with improvements in Doppler ultrasound technology, but non-palpable varicoceles diagnosed by ultrasound alone are considered sub-clinical. While subclinical varicoceles are frequently

detected on ultrasonography, physical examination remains the gold standard for detection of varicoceles, as correction of subclinical varicoceles has not been shown to impact subsequent fertility. Nonetheless, while subclinical varicoceles are frequently detected on ultrasonography, the physical examination remains the gold standard as correction of subclinical varicoceles has not been shown to impact subsequent fertility.[2] In contrast, clinical varicocele repair has been shown to improve spermatogenesis, prevent further testicular injury and increase serum testosterone.[3-5]

Varicoceles are defined by a grading system, and the success of repair is in part dependent on the preoperative grade of varicocele.[5, 6] As described above, subclinical varicoceles are detected solely by ultrasonography, and are not palpable. Grade I varicoceles are only palpable when a Valsalva maneuver is performed. Grade II varicoceles are large enough to be detected by palpation without the Valsalva maneuver, while grade III varicoceles are visible through the scrotal skin while at rest and do not require abdominal straining for diagnosis. The larger the varicocele, the more likely that it is associated with impairment in semen quality and that subsequent repair will significantly improve semen quality.

Treatment Indications

The American Society of Reproductive Medicine and the American Urological Association jointly published best practice policies for the treatment of infertile men with varicoceles.[7] Patients are candidates for surgical repair when each of the following conditions are met: the varicocele is palpable on physical examination, or if suspected but not palpated, corroborated via ultrasound examination; an abnormal semen analysis is noted; and the female partner has normal fertility or a potentially correctable cause of infertility.[7] Adolescents should be considered for repair if demonstrable reduction in testicular volume is noted on the side of the varicocele. Furthermore, men with a varicocele and abnormal semen parameters and a concurrent desire for future conception may also be considered for repair. In addition to the fertility indications for repair, patients with large varicoceles may on occasion have significant scrotal discomfort that also may benefit from treatment.

Surgical Approaches

Scrotal Approach

The scrotal approach was historically the first utilized, but has since become obsolete due to risk of injury to other cord structures and the complexity of the testicular pampiniform plexus, making identification and ligation of the complete venous structure difficult. The availability and comparative simplicity of other approaches have led to the consideration of the scrotal approach for historical purposes only.

Retroperitoneal Approach

The retroperitoneal approach to varicocele repair involves isolating and ligating the internal spermatic vein above the level of the internal inguinal ring. With the patient in the supine position, an abdominal incision is made two fingerbreadths inferomedial to the anterior superior iliac spine, approximately 2 cm above the internal inguinal ring (Figure 6.1). The incision is next carried down through Camper's and Scarpa's fascia to the level of the external oblique aponeurosis. The aponeurosis is incised in the direction of its fibers, the internal oblique muscle is split (Figure 6.2), and the exposed transversus muscle and transversalis fascia are incised (Figure 6.3). Retraction of the inferior external oblique fascia allows identification of the internal ring. The peritoneum is reflected medially, and the spermatic vessels can be identified, often associated with the peritoneal reflection (Figure 6.4). The spermatic veins are individually ligated, and the spermatic artery is identified and preserved (Figure 6.5). Generally, two to three veins are ligated in this area. Following ligation, the incision is closed in two layers with absorbable suture.

Laparoscopic Retroperitoneal Approach

Laparoscopic varicocele repair utilizes the same approach and surgical principles as the open retroperitoneal procedure. This approach should be performed only by surgeons experienced in laparoscopic techniques due to the risk of significant injury to the bowel or surrounding vascular structures. After placement of a Foley catheter and a nasogastric tube, a laparoscopic 12 mm trochar is placed immediately below the umbilicus. A second 12 mm port is placed on the contralateral side just lateral to the rectus muscle and below the umbilicus. Finally, a

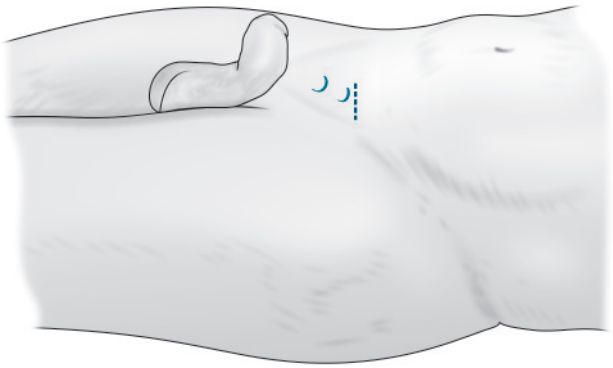


Figure 6.1: Incision is made inferomedial to the anterior superior iliac spine and 2 cm above the internal inguinal ring

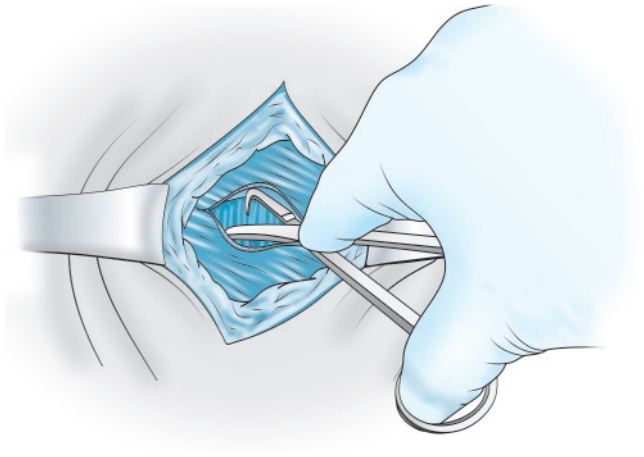


Figure 6.2: The external oblique aponeurosis is incised and the internal oblique muscle is split

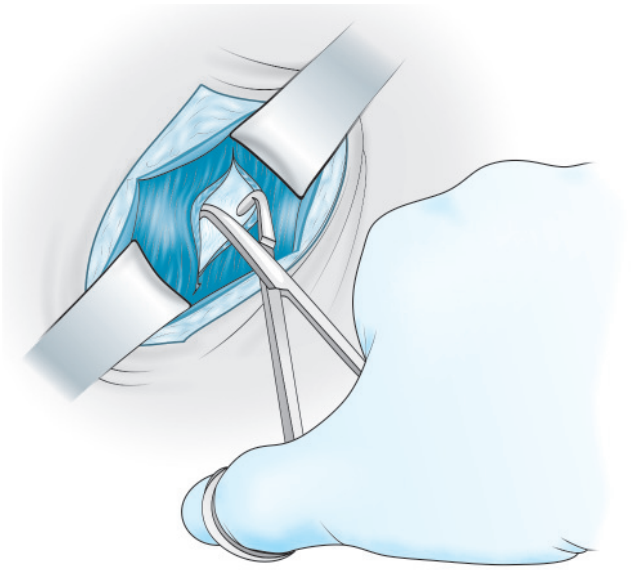


Figure 6.3: Transversus muscle and transversalis fascia are incised

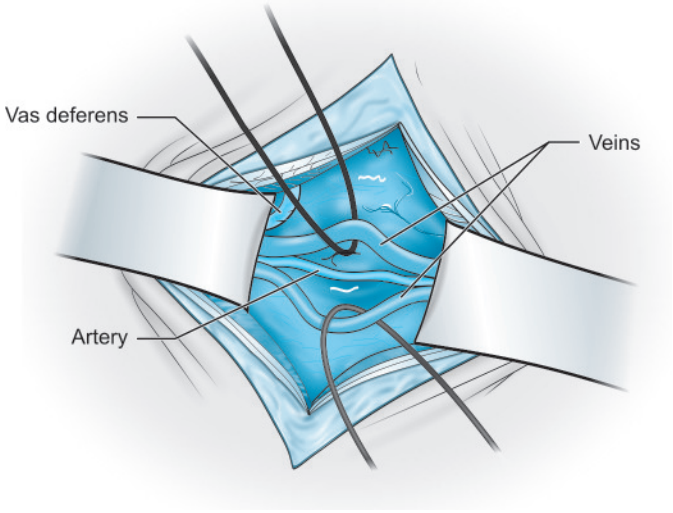


Figure 6.4: Peritoneum reflected, with identification of the spermatic vessels

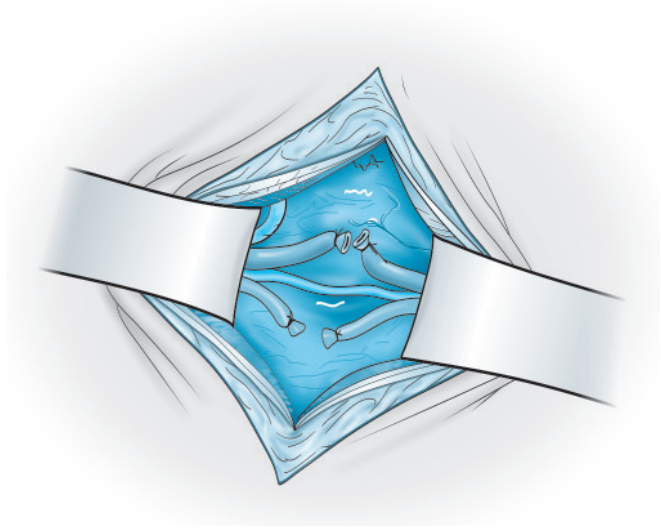


Figure 6.5: Spermatic artery is identified and preserved and the spermatic veins are ligated

5 mm port is placed to the left of the umbilicus. The patient is placed in the Trendelenburg position. The camera is inserted through the 12 mm umbilical port, and the key anatomic structures are identified. If there is difficulty identifying cord structures, traction on the testis can identify the vessels. The parietal peritoneum is incised lateral to the spermatic cord structures exiting the internal ring, and the testicular artery and veins are dissected and isolated. The veins are isolated and ligated with hemostatic surgical clips and transected. The ports are removed under direct vision after hemostasis is assured, and the fascial layer is closed, followed by closure of the

skin. Potential complication of laparoscopic dissection of the spermatic vessels involves complete ligation of all spermatic vessels, including the testicular artery. Although the collateral blood supply to the testis through the cremasteric and vasal arteries is usually sufficient to prevent testicular atrophy, this cannot be predicted reliably. Inadvertent ligation of lymphatics during laparoscopic repair can also result in postoperative persistent hydrocele. Laparoscopy should be considered only by those comfortable with the technique, as the open approach is still considered the gold standard retroperitoneal approach.

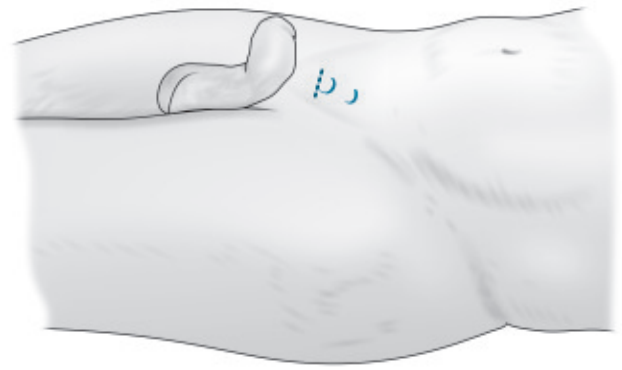


Figure 6.6: Incision made just below the level of the external ring

Subinguinal/Inguinal Approaches

The majority of urologic surgeons perform varicocele repair using microsurgical techniques to preserve the internal spermatic arteries as well as lymphatics, which play a critical role in preventing postoperative persistent hydrocele. The inguinal or subinguinal approaches are preferred for microsurgical dissection, and the specific indications for each will be discussed here. Since no fascial or abdominal muscle incisions are required, the subinguinal approach offers patients a significantly quicker return to normal activity over the inguinal or retroperitoneal approaches.

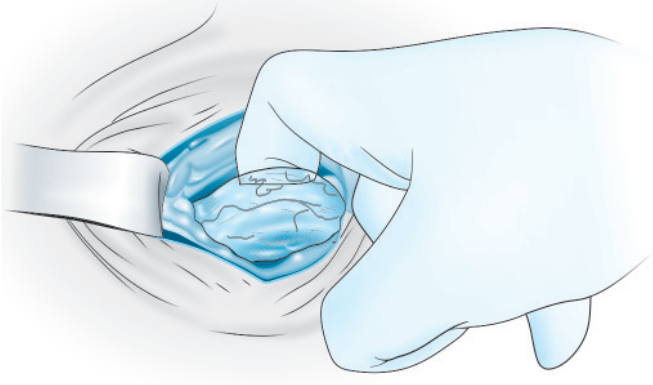


Figure 6.7: Penrose drain is passed under mobilized cord

Subinguinal Approach

The subinguinal approach for varicocele repair was popularized with the assistance of the operating microscope, allowing more accurate identification and exclusion of the cord lymphatics or testicular artery to prevent accidental ligation. A 2 to 3 cm transverse incision is made just below the level of the external ring (Figure 6.6). Scarpa's fascia is opened, and the loose connective tissue overlying the spermatic cord is incised at the level of the external ring. The cord is mobilized and lifted out of the incision, and a Penrose drain is passed under it (Figures 6.7 and 6.8). For microsurgical dissection, the microscope is brought into the operating field at this juncture. The cremasteric muscle is opened in the direction of its fibers to expose cord structures (Figure 6.9). The position of the testicular artery can be identified through either visualization or using a 2 mm Doppler ultrasound probe. All spermatic veins encountered are carefully isolated from surrounding structures by passing a vessel loop or small Penrose drain around each vein as they are encountered (Figure 6.10). The vasal structures

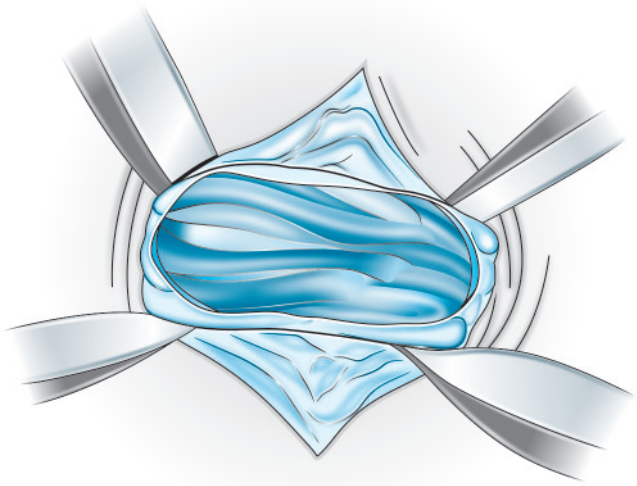


Figure 6.8

are carefully avoided. Veins found to be adherent to an artery require careful dissection to maintain arterial integrity. Advancing the dissection proximally can help identify a venous target for ligation, as the veins coalesce proximally to form larger veins that course away from

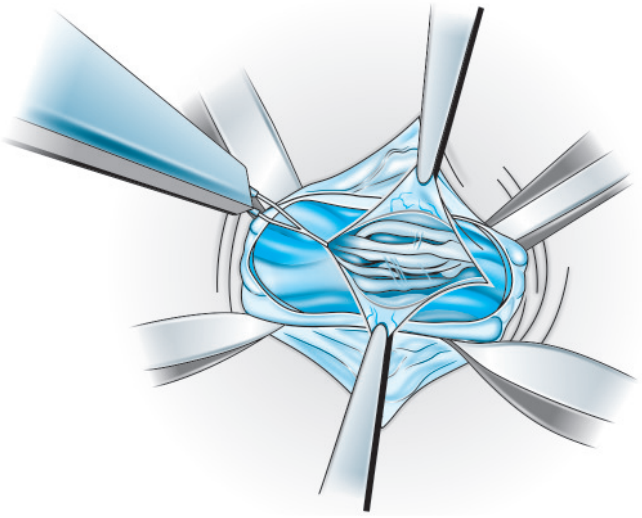


Figure 6.9: Cremasteric muscle is opened in the direction of its fibers

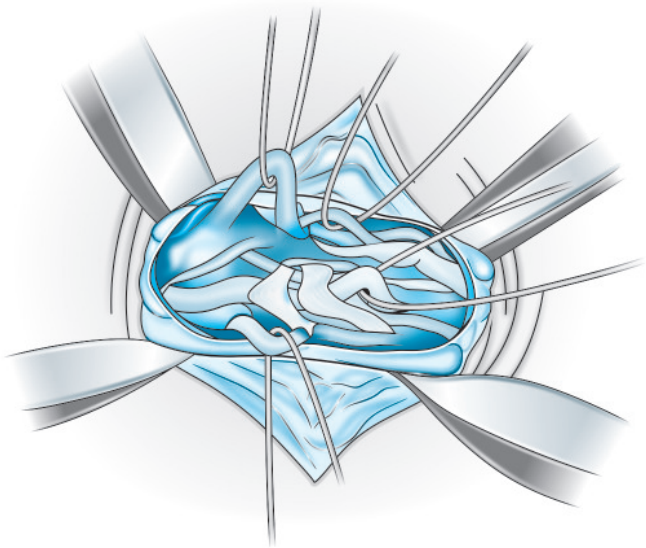


Figure 6.10: Spermatic veins are isolated by passing a vessel loop around each vein as they are encountered

the artery. All target veins are ligated with 4-0 or 5-0 permanent suture and transected (Figure 6.11). The cord is then returned to its normal position, and the incision is closed.

Inguinal Approach

The inguinal approach can be used in almost any patient. It allows for mobilization of the cord, identification of veins within the cremasteric muscle and identification of veins perforating the posterior inguinal canal. A 3 to 4

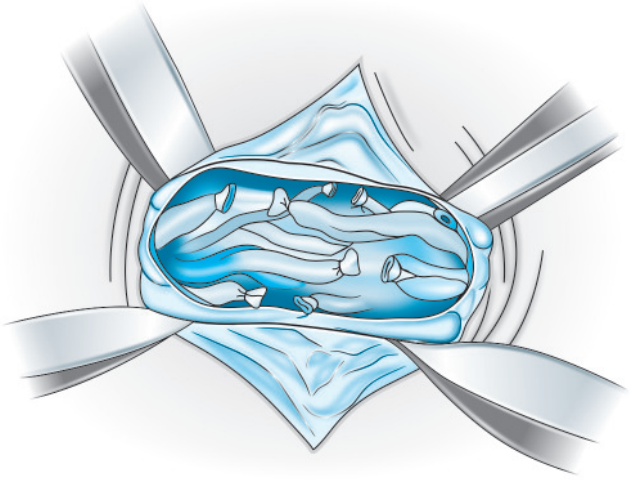


Figure 6.11: Target veins are ligated with 4-0 or 5-0 permanent suture and transected

cm oblique incision is made parallel to the inguinal ligament as one would do for standard inguinal hernia repair (Figure 6.12). The incision is carried down to the external oblique aponeurosis, which is incised in the direction of its fibers (Figure 6.13), and the ilioinguinal nerve is identified and preserved. The incision should be carried through the external ring to allow complete mobilization of cord. The cord is removed from the inguinal canal to be isolated with a Penrose drain. For microsurgical dissection, the microscope is brought into the operating field at this juncture. The cremasteric muscle is opened in the direction of its fibers to expose cord structures. As in the subinguinal approach, all spermatic veins are meticulously isolated by passing a vessel loop around each vein as they are encountered (Figure 6.14). The remainder of the procedure is performed as described in the subinguinal approach.

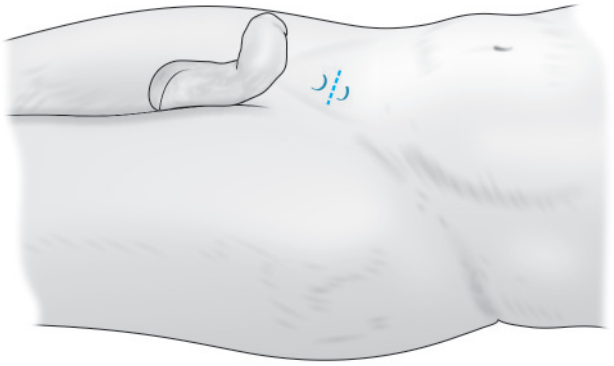


Figure 6.12: Incision made parallel to the inguinal ligament

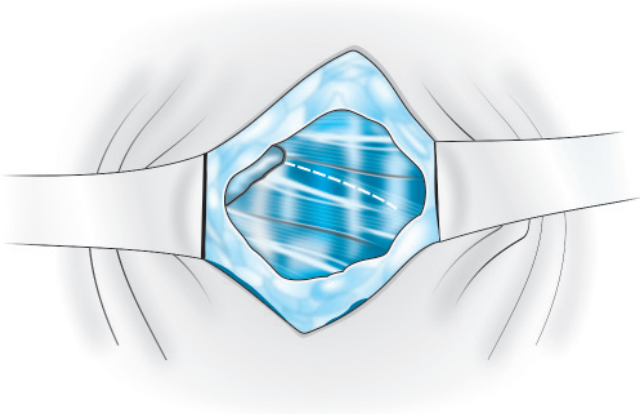


Figure 6.13: Incision carried down to the external oblique aponeurosis

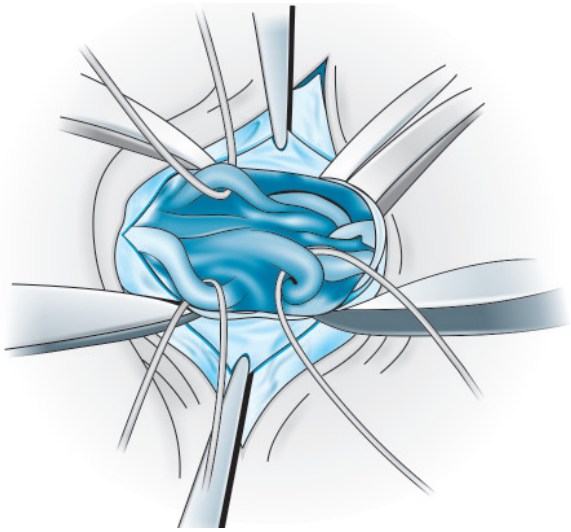


Figure 6.14: Spermatic veins meticulously isolated when encountered

Microsurgical versus Non-microsurgical Approaches

The improved visualization with microsurgical dissection allows significant reduction in postoperative complications, such as testicular artery injury, hydrocele formation and varicocele recurrence. The recurrence rate for microscopic inguinal varicocelectomy has been reported between 1% and 2%, compared with 9% and 16% for non-microscopic inguinal varicocele repair.[8] These results are directly attributed to the improved ability to visualize the veins, artery and lymphatics.

Percutaneous Embolization

Transvenous sclerotherapy for the ablation of varicocele was first reported more than 30 years ago. Today, embolization of the spermatic veins can be accomplished with coils, balloons or sclerotherapy but requires a skilled interventional radiologist to be considered a valid alternative to surgical intervention. Percutaneous varicocele embolization is especially useful in a recurrent or persistent varicocele, when radiological studies can help identify aberrant anatomy.[9] The coils or balloons are placed under local anesthesia and involve a cut-down incision over the femoral or internal jugular vein. A catheter is passed into the internal spermatic veins to allow deployment of the coils or balloons and the embolization is completed. Complications associated with percutaneous embolization include balloon deflation and migration and varicocele recurrence.

Complications

Hydrocele

The most common complication after non-microsurgical varicocelectomy is hydrocele formation, likely due to unrecognized injury or ligation of the lymphatic structures, and lymphatic obstruction is the most likely cause of hydrocele formation. The rate of hydrocele occurrence following non-microsurgical varicocele repair is approximately 7%.[10] Conversely, the rate of hydrocele formation following microsurgical varicocelectomy is much lower, with several recent studies reporting no patients with postoperative hydrocele[10,11] offering further support for microsurgical techniques.

Varicocele Recurrence

Rates of varicocele recurrence or persistence can be considered based on the approach utilized during varicocelectomy. Microsurgical approaches maximize surgical efficacy for varicocele repair, with a recurrence rate for microscopic inguinal varicocelectomy noted to be 1 to 2%.[10,11] Conversely, non-microscopic inguinal varicocele repair report recurrence rates from 9 to 16%,[8,10] and high retroperitoneal varicocele repairs offer recurrence rates of 11 to 15%.[12]

Testicular Artery Injury

In a recent study of 2102 patients undergoing microsurgical varicocelectomy, only 19 (0.9%) experienced testicular artery injury during microsurgical varicocelectomy, with only one patient developing testicular atrophy.[13] Thus, the overall risk of testicular

atrophy is quite low. In general, testicular artery injury does not always lead to testicular atrophy due to the redundant vascular supply from the vasal and cremasteric arteries.

Follow-up

Patients should be followed to monitor incision healing in the first 1-2 weeks. Semen analysis should be performed 4 months after varicocele repair. In addition, regular monitoring for at least 1 year or until the time of conception is also necessary to confirm improved semen analysis. Care should also be taken to monitor for possible complications during follow-up as well.

REFERENCES

1. Nagler HM, Martinis FG. Varicocele In: Lipshultz LI, Howards SS, (Eds) Infertility in the Male, 3rd ed.;1997;336-59 St. Louis: Mosby.
2. Jarow JP, Ogle SR, Eskew LA. Seminal improvement following repair of ultrasound detected subclinical varicoceles J Urol 1996;155:1287-90.
3. Agarwal A, Deepinder F, Cocuzza M, Agarwal R, Sabanegh ES, Jr. Short R, et al. Effects of Varicocelectomy on Improvement in Semen Parameters: New Meta-analytical Approach. Urology 2007;70:532.
4. Marmar JL, Agarwal A, Prabakaran S, Agarwal R, Short RA, Benoff S, Thomas AJ Jr. Reassessing the value of varicocelectomy as a treatment for male subfertility with a new meta-analysis. Fert Ster 2007;88:639-48.
5. Shindel AW. Yan Y. Naughton CK. Does the number and size of veins ligated at left-sided microsurgical subinguinal varicocelectomy affect semen analysis outcomes? Urology 2007;69:1176-80.
6. Schiff JD, Li PS, Goldstein M. Correlation of ultrasound-measured venous size and reversal of flow with Valsalva with improvement in semen-analysis parameters after varicocelectomy. Fert Ster 2006;86:250-2.
7. Practice Committee of the American Society for Reproductive Medicine. Report on varicocele and infertility. Fert Ster 2006;86:S93-5.
8. Cayan S, Kadioglu TC, Tefekli A, et al. Comparison of results and complications of high ligation surgery and microsurgical high inguinal varicocelectomy in the treatment of varicocele. Urology 2000;55:750-4.
9. Mazzoni G, Minucci S, Gentile V. Recurrent varicocele: role of antegrade sclerotherapy as first choice treatment. Eur Urol 2002;41:614-8.
10. Al-Kandari AM, Shabaan H, Ibrahim HM, Elshebiny YH, Shokeir AA. Comparison of outcomes of different varicocelectomy techniques: open inguinal, laparoscopic, and subinguinal microscopic varicocelectomy: a randomized clinical trial. Urology 2007;69(3):417-20.
11. Carbone Jr. DJ, Merhoff V. Complication rate of microsurgical varicocele ligation without delivery of the testis. Arch Androl 2003;49:201-4.
12. Niedzielski J, Paduch DA. Recurrence of varicocele after high retroperitoneal repair: Implications of intraoperative venography. J Urol 2001;165:937-40.
13. Chan PT, Goldstein M. Incidence and postoperative outcomes of accidental ligation of the testicular artery during microsurgical varicocelectomy. J Urol 2005;173: 482-4.

CHAPTER 7

Endocrine Disorders: Diagnosis and Management

Philip Kumanov

ABSTRACT

Male factor infertility affects approximately 40% of the infertile couple. A number of different causes can affect the semen parameters, causing similar effects. Etiology of male infertility is difficult to understand and even more difficult to treat.

Diagnosis of endocrine disorders affecting male infertility and the treatment of these disorders is a challenging task but bear good promise for induction of natural pregnancies as well as assisted reproduction. This chapter focuses on male endocrine disorders along with their diagnosis and management.

Key words: Male hypogonadism, testosterone, azoospermia, gonadotrophins.

MALE HYPOGONADISM

Definition and Prevalence

The inability of testes to produce physiologic amounts of testosterone, spermatozoa, or both. Hypogonadism can be a result of hypothalamic and/or pituitary dysfunction (secondary or hypogonadotrophic) or can be due to testicular failure (primary or hypergonadotrophic). Hypogonadism may be congenital or acquired. Hypogonadism affects up to 20% of males.[9]

Clinical Presentation

Clinical features do not depend upon the level (hypothalamic, hypophyseal or testicular) of dysfunction but rather on subnormal androgen production or action. Age at onset and severity are crucial for the clinical presentation.

Androgen deficiency in the first trimester of fetal development results in male pseudohermaphroditism with genital ambiguity. Later in the prenatal period, deficient androgen production and/or action may lead to cryptorchidism as well as micropenis.

Prepubertal male hormonal deficiency causes insufficient sexual development with small penis and testes and eunuchoid skeletal proportions (arm span 5 cm or greater than height and lower segment greater than upper segment). The voice remains high-pitched; muscle mass and strength are subnormal; pubic and axillary hair is sparse, while facial and trunkal is absent. The affected men are infertile.

Postpubertal development of androgen deficiency does not affect body proportions or penis size. The main complaints are diminished libido, erectile dysfunction, infertility and decreased strength and energy. Facial and body hair growth decrease only in patients with long-standing postpubertal hypogonadism.[4]

Male Gonadal Disorders

Male hypogonadism may be subdivided into three general categories[4] (Table 7.1).

Table 7.1: Classification of male hypogonadism

Secondary or hypogonadotrophic hypogonadism
Panhypopituitarism
Isolated LH deficiency (fertile eunuch syndrome)
Isolated FSH deficiency
LH and FSH deficiency
a. With normal sense of smell-idiopathic hypogonadotrophic hypogonadism (IHH)
b. With hyposmia or anosmia - Kallmann's syndrome

Contd...

Contd...

- c. With neurologic disturbances
  - Prader-Labhart-Willi syndrome
  - Laurence-Moon-Biedl syndrome
  - Möbius' syndrome
  - Carpenter's syndrome
  - Rud's syndrome
  - Cerebellar ataxia
- d. Hemochromatosis
- e. Hyperprolactinemia
- f. Malnutrition

Primary or hypergonadotrophic hypogonadism
- Klinefelter's syndrome
- XX male syndrome
- Bilateral anorchia (vanishing testes syndrome)
- Noonan's syndrome
- Myotonic dystrophy
- Adult seminiferous tubule failure
- Varicocele
- Defects in androgen biosynthesis

Defects in androgen action
- Complete androgen insensitivity (testicular feminization)
- Incomplete androgen insensitivity

Diagnosis

In the history, attention is paid to data about delayed puberty, infections (mumps), abdominal or genital trauma, testicular torsion and sense of smell. Information about infertility or anosmia in relatives is also important.

Clinical examination: The presence of hypospadias, epispadias or small penis should be noted. The fully stretched dorsal length of the flaccid penis should be measured from the pubopenile skin junction to the tip of the glans. Males with penile length below 7.5 cm require treatment.[11] The easiest way for estimation of testicular volume is the comparative palpation with the Prader orchidometer. According to this method, healthy adults have volumes greater than 10-12 ml. Subnormal volume indicates underdevelopment or regression of the seminiferous tubules. Therefore, assessment of testicular volume as well as consistency is important in the evaluation of males with infertility. Presence of varicocele should not be neglected. The patient must be examined in the upright position while performing the Valsalva maneuver.

After puberty, semen analysis is very useful. A normal result of at least two semen samples excludes hypogonadism.

Because of the circadian variations in secretion, serum testosterone levels must be measured in blood samples obtained between 7:00 and 11:00 am.[7] The determination should be done in more than one blood sample. Total serum testosterone levels above 12 nmol/l (346 ng/dL) exclude hypogonadism. In some laboratories, the lower limit of the normal range for total testosterone level in healthy young men is 10.4 nmol/l (300 ng/dl).[2] Values below 8 nmol/l confirm the diagnosis of hypogonadism and require substitution.[7] In most cases it is not necessary to determine free testosterone level, but in some situations such as hypo- and hyperthyroidism, obesity, acromegaly or hyperinsuli-nemia, as well as in aging males, alterations in the levels of sex hormone-binding globulin (SHBG) may occur with subsequent disruption of the correlation between total and free testosterone levels. The lower limit of the normal range for serum free testosterone is 0.17 nmol/l (50 pg/mL).[2] Free testosterone levels above 0.25 nmol/L (72 pg/mL) do not require substitution.[7]

Measurements of basal follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations are useful to distinguish between hypergonadotrophic hypogonadism, in which either or both of the gonadotrophins are elevated, and hypogonadotrophic hypogonadism, in which the gonadotrophins are low or inappropriately normal in the presence of decreased testosterone secretion.[4] Each laboratory should have its own reference range. FSH and LH are not helpful in aging males.

Measurement of prolactin serum levels should be undertaken in patients with hypogonadotrophic hypogonadism.

Recently the development of an accurate solid-phase sandwich enzyme-linked immunosorbent assay for measurement of inhibin B serum levels resulted in the ability to use serum levels of this hormone for the evaluation of infertile men, pubertal development and of the effects of gonadotrophin therapy.[6] Serum levels higher than 100 pg/mL are indicative of normal seminiferous tubules.

Human chorionic gonadotrophin (hCG) demonstrates biologic actions similar to those of LH. It stimulates the synthesis and secretion of testicular steroids. Following administration of hCG (intramuscular injection of 4000 IU daily for 4 days or alternatively, a single dose of 5000 IU/1.7 m^2 in adults and 100 IU/kg in children) patients with hypothalamic or pituitary dysfunction will have a normal reaction (a doubling of the testosterone level), while those with hypergonadotrophic hypogonadism will have a blunted testosterone response.

A relatively high number of healthy males have LH and FSH levels that are below the lower limit of the normal range or even undetectable. Therefore, stimulation of pituitary gonadotrophin secretion may be reasonable in some cases. The test utilizing decapeptide gonadotrophin-releasing hormone (GnRH) is performed by administering 100 µg of the substance by rapid intravenous bolus. Blood samples for FSH and LH measurements are obtained at 0, 15, 30, 60 and 90 minutes. A two- to fivefold (or even more) increase in LH over baseline levels with the peak values at 15 or 30 minutes and a twofold rise of FSH at 60 or 90 minutes should be regarded as a normal response in adult males. Unfortunately, measurement of LH and FSH following the single administration of GnRH is not always helpful in distinguishing between hypothalamic and hypophyseal dysfunction: patients with long-standing hypothalamic disorders may not show a response to a GnRH test even if the pituitary is unaffected. Therefore, the test is performed after repeated injections of GnRH. Responsiveness to the releasing hormone may be restored in patients with hypothalamic dysfunction, but this does not occur in patients with pituitary insufficiency.

Clomiphen citrate as a nonsteroid compound with weak estrogenic activity binds to estrogen receptors in the hypothalamus and thus inhibits the gonadal feedback control of the hypothalamus and pituitary, mediated normally by estradiol. The consequence is an increase of GnRH and subsequently of LH and FSH. A normal response to clomiphen citrate, 100-200 mg daily, given orally for 7 days, is a twofold increase in LH and FSH, measured on days 0, 4, 7 and 10.[9] Subnormal increase indicates hypothalamic or pituitary dysfunction but does not differentiate between these two situations.

Chromosomal analysis is indicated in patients with elevated gonadotrophins to distinguish between genetic abnormalities (e.g. Klinefelter's syndrome) and acquired testicular defects.

Testicular biopsy is no longer used for routine evaluation of hypogonadism. It is indicated in men with normal-sized testes and azoospermia to differentiate between spermatogenic failure and ductal obstruction. However, it is also possible to distinguish between these two situations by the measurement of serum FSH and inhibin B concentrations.

The most important clinical features and laboratory tests for each lifestage are given in Figure 7.1.

Treatment

Androgen Replacement Therapy

Serum total testosterone levels below 8 nmol/L (231 ng/dL) or free testosterone below 0.17 nmol/L (50 pg/mL) require substitution. Since symptoms of androgen deficiency become manifest between 12 and 8 nmol/L, treatment can be considered in those in who alternative causes of these symptoms have been excluded.[7]

Testosterone formulations include testosterone enanthate or cypionate, the scrotal testosterone patch, a non-genital transdermal system, testosterone gel, buccal testosterone, oral testosterone undecanoate, injectable long-acting testosterone undecanoate in oil and testosterone pellets.[2]

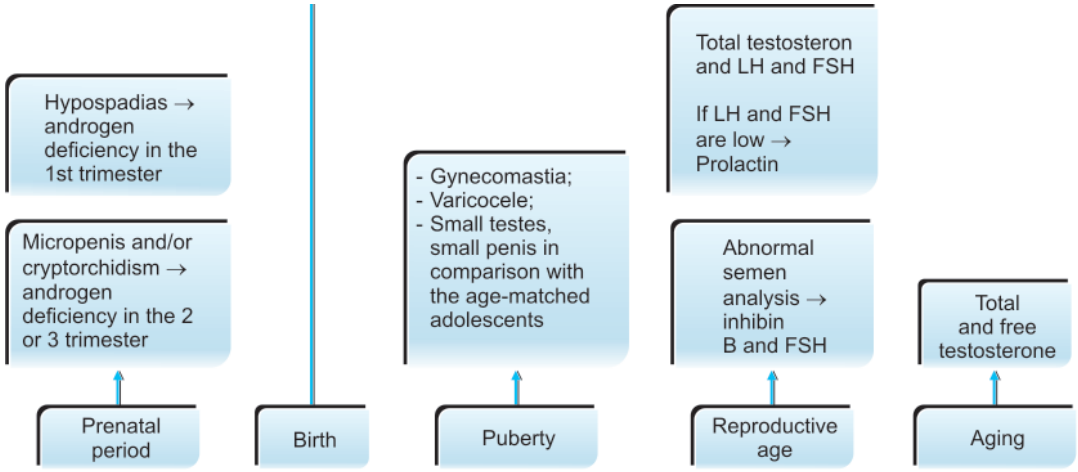


Figure 7.1: Clinical features and laboratory tests in the different life periods of the men (Ph. Kumanov—unpublished)
Note: In the aging males gonadotrophic hormones are not helpful

Testosterone replacement therapy induces virilization in the hypogonadal male, restores libido and erectile function and improves muscle mass and strength. Hypogonadism is a risk factor for osteoporosis. Testosterone administration in men with subnormal androgen production improves bone mineral density. This therapy also has favourable effects on mood and well-being.

Androgens stimulate prostatic growth and may promote the growth of an existing cancer. They also stimulate erythropoiesis and may cause a fall in HDL cholesterol levels. Side effects include acne, gynecomastia, fluid retention and exacerbation of obstructive sleep apnea. Prostate cancer and breast cancer are absolute contraindications for testosterone treatment. Relative contraindications include polycythemia, sleep apnea, benign prostate hyperplasia and cardiac failure.

Therapy should be monitored by measurement of testosterone levels, hemoglobin and hematocrit and serum lipids, as well as digital rectal examination and prostatic specific antigen (PSA) quarterly for 1 year and then annually.

Treatment with Gonadotrophins or GnRH

Testosterone replacement therapy does not restore fertility. Men with hypogonadotrophic hypogonadism who desire children may be treated with gonadotrophins to initiate and maintain spermatogenesis. Testosterone treatment should be stopped before initiating gonadotrophin therapy. Men with hypergonadotrophic hypogonadism will not respond to gonadotrophin or GnRH therapy. Cryptorchidism does not preclude patients from gaining fertility, especially if it is unilateral.[3]

HCG (Profasi^{R}, Pregnyl^{R}) 1000-2500 IU is given twice per week. Both intramuscular and subcutaneous administration is effective. Most patients also require FSH (Follistim^{R}, Puregon^{R}, GonalF^{R}) 75-150-300 IU 3 times weekly. Once it is initiated, sperm production often can be maintained with hCG alone.[3] Alternatively, pulsatile GnRH can be administered subcutaneously every 120 minutes using a portable minipump. A starting dose of 4 µg per pulse should be increased with 2 µg every 2 weeks if LH secretion does not rise. Maximum dose is 20 µg per pulse.[3] It may take approximately two years, until sperm appear in the ejaculate or a pregnancy has been induced. Testicular size, testosterone and estradiol levels, as well as hemoglobin and hematocrit, should be monitored. The main side effect of treatment with gonadotrophins is gynecomastia.

Cryptorchidism

Definition and Prevalence

Cryptorchidism is the unilateral or bilateral absence of the testes from the scrotum due to failure of normal testicular descent from the genital ridge through the external inguinal ring. Between 2 and 5% of full-term male infants have cryptorchidism. In more than 2/3 of cases with cryptorchidism noted at birth, spontaneous testicular descent occurs in the first three months of life; thereafter the incidence decreases further, so that by the end of the first year of life it is 0.2-0.8%. Approximately 0.2-0.3% of adult males are cryptorchid.

Unilateral cryptorchidism is up to 10 times more common than bilateral maldescended testes. Right-sided cryptorchidism is more common than left-sided cryptorchidism, possibly because normally the descent on the left side occurs earlier. Approximately 2/3 of cryptorchid testes are located in the inguinal canal or in a high scrotal position; the rest are intra-abdominal or ectopic.

Etiology and Pathology

Testicular descent occurs in two phases. Normally between the 10th and 15th week of fetal development the gonad reaches the internal ring of the inguinal canal, probably under the influence of insulin-like factor 3; the migration to the final position in the scrotum occurs after the 26th week and is mediated by androgens.[1] In addition to the hormonal influence, mechanical factors also play a role in this process.

Cryptorchidism is common in patients with defects in androgen synthesis or action, as well as in patients with congenital gonadotrophin deficiency. The primary unresolved question is whether pathologic changes in the gonads are due to the effects of cryptorchidism, or on the contrary, intrinsic abnormalities in the testes lead to the failure of the descent. It is suggested that the extrascrotal environment, especially increased temperature, is at least partly responsible for the depression of spermatogenesis. Histologic studies have demonstrated a decrease in the size of seminiferous tubules and number of spermatogonia. Such changes have been detected in the first months after birth.

More severe abnormalities are observed in intra-abdominal testes than in inguinal testes. In cases with unilateral cryptorchidism, pathologic changes and an increased risk for development of germ cell neoplasms are also established for the normally descended scrotal testis. Obviously, the adverse changes in the testes depend on the location of the undescended gonad and the duration of the cryptorchidism.

Diagnosis and Differential Diagnosis

Absence of one or both testes is the main clinical finding. The child must be examined in a warm room with warm hands. Retractile testis (pseudocryptorchidism) is due to the cremasteric reflex, which is most prominent in childhood. The retractile testis can be manipulated into the scrotum with gentle palpation and pressure over the abdomen, which is not possible with the true cryptorchid gonad. FSH, LH and testosterone are not helpful for evaluation of prepubertal boys with unilateral cryptorchidism. However, they are useful in differentiating cryptorchid patients from those with bilateral anorchia. The lattest is characterized by high gonadotrophins, low serum testosterone and blunted or absent testosterone response following hCG stimulation.

Adults may have oligo- and or asthenozoospermia and even azoospermia in cases with a history of bilateral cryptorchidism. A low serum inhibin B level and elevated basal serum FSH concentration or an exaggerated FSH increase following GnRH administration are all characteristic of these findings. Magnetic resonance imaging (MRI) may be also helpful for the evaluation of cases with intra-abdominal testes.

Complications and Consequences

A high percentage of cryptorchid patients has ipsilateral inguinal hernia because of the failure of the processus vaginalis to close. Torsion may occur. Cryptorchid testes are susceptible to trauma, and the risk for malignant degeneration is increased, particularly in patients with intra-abdominal testes versus those with inguinal testes. Cryptorchid children may have problems with gender identity.

If left untreated, bilateral cryptorchidism is associated with infertility in 75% of cases, and 50% of patients with unilateral undescended testicle are infertile. Even when the abnormality is corrected early in life, i.e. long before puberty, many of the affected men have infertility.

Treatment

Optimal time for treatment is around the age of 2 years. hCG 500 IU (in infants) and 1000 IU (in older children) intramuscularly should be given 2 times a week for 5 weeks. The treatment schedule may be repeated after a pause of 8-12 weeks. hCG is not indicated in cases with ectopic testes and in those with associated inguinal hernia.

This treatment is successful in less than 25% of patients, primarily in those with unilateral inguinal or high scrotal testis. Equal results are achieved with GnRH given 3 times a day by nasal spray for 4 weeks. Orchiopexy is the treatment option for various cases with maldescended testes.

Androgen Insensitivity Syndromes

Definition and Prevalence

Androgen insensitivity causes undermasculinization of various degrees in 46 XY individuals.

Complete androgen insensitivity is rare with an incidence of between 1: 20 000 and 1: 60 000 live-born males. The incidence of partial forms is not known.

Pathogenesis

The androgen receptor gene is located on the X chromosome between Xq11 and Xq13. Androgen insensitivity syndromes result from defects in androgen receptor number or function. A large number of mutations have been described, inherited in an X-linked recessive fashion.

Clinical Presentation

Androgen insensitivity may be complete or partial (incomplete).

Complete androgen insensitivity syndrome (CAIS) (testicular feminization) is characterized by female external genitalia, testes located abdominally or in the inguinal canal, absent or hypoplastic Wolffian ducts, absent or rudimentary Müllerian derivates (due to the unimpaired action of nonsteroidal anti-Müllerian hormone, secreted by the fetal Sertoli cells) with a blind vaginal pouch in a 46 XY individual. Breasts develop at puberty, but menarche does not occur. Pubic and axillary hair is usually sparse but may be absent (hairless women). Gender identity is female.

Partial androgen insensitivity presents with a wide spectrum from normal male phenotype with infertility to individuals with genital ambiguity. Coexistence of hypospadias and gynecomastia is not unusual.

Diagnosis

CAIS may be suggested before puberty by the presence of bilateral masses in the inguinal canals or labia and postpubertally by primary amenorrhea in phenotypic females with sparse or absent axillary and pubic hair and absence of a uterus by pelvic ultrasound examination. Patients with CAIS as well as those with partial androgen insensitivity exhibit elevated serum testosterone and LH levels after puberty, but FSH is normal. Estradiol levels are higher than in normal males. Failure of SHBG to decrease after testosterone administration confirms the androgen insensitivity. Karyotyping reveals 46, XY and is indicated, especially in females with bilateral inguinal hernias. Androgen receptor studies are helpful in cases with incomplete insensitivity.

Treatment

Treatment of patients with CAIS consists of castration after puberty with subsequent estrogen replacement. Orchidec-tomy should be performed because of an increased risk of gonadal neoplasm development with age.

Hyperprolactinemia

Causes

Micro- and macroprolactinomas; pituitary adenomas, secreting both prolactin and growth hormone; empty sella syndrome; processes causing pituitary stalk compression or section (meningiomas, cranioph-aryngiomas, sarcoidosis, head injury). Primary hypothyroidism via TRH hypersecretion increases prolactin. Chronic renal failure reduces prolactin clearance. Hyperprolactinemia also can be caused by chronic (mis-)use of many drugs such as opiates, neuroleptics, antidepressants, dopamine receptor antagonists, antihypertensives, protease inhibitors, H_2 antagonists and omeprazole.

Pathogenesis

Two possible pathways by which hyperprolactinemia disturbs gonadal function have been suggested: by affecting the secretion of LH and FSH and/or by impeding their action on the gonads.

Clinical Presentation

Loss of libido and/or erectile dysfunction is the main symptom. In a recently published study of 165 men with erectile dysfunction, 16 patients (9.7%) had hyperprolacti-nemia – 10 had pituitary adenomas and 6 had drug-induced hyperprolactinemia.[10]

Reduced fertility as well as gynecomastia is unusual. Galactorrhea is rare in men, occurring in 10-20%. Macroprolactinomas in males tend to be more frequent than in the female population probably due to non-specific symptoms such as erectile dysfunction that delay establishment of the appropriate diagnosis.

Diagnosis

A history of drug intake must be obtained. The stress may cause mild hyperprolactinemia. Prolactin measurement is indicated in any man with erectile dysfunction as well as in cases with hypogonadotrophic (secondary) hypogonadism.

Basal prolactin levels, measured in two to three blood samples, gonadotrophins, testosterone and thyroid-stimulating hormone (TSH) serum concentrations should be checked. Serum prolactin levels higher than 3000 mU/L are suggestive of a tumor.[9] Currently available suppression and stimulation tests do not distinguish prolactin-secreting tumors from other causes of hyperprolactinemia.[4] Therefore, the diagnosis must be established by the assessment of basal prolactin levels. In addition, MRI should be performed, and liver and kidney function should be assessed.

Treatment

Administration of a dopamine agonist is first-line treatment in tumors of all sizes. It leads to prolactin suppression in most patients, tumor shrinkage and subsequent restoration of gonadal function. Historically, bromocriptine was the first effective medical therapy. However, cabergoline, a nonergot dopamine agonist administered once or twice a week, is longer-acting and better-tolerated.

Transsphenoidal microsurgery is the surgical procedure of choice in patients with prolactinomas and is indicated only for those who are intolerant of dopamine agonists or are resistant to them. Radiation therapy is

reserved for patients in whom surgery and dopamine agonists failed to control their pituitary adenomas. Drug-induced hyperprolactinemia is usually reversible after stopping the relevant medication and substituting a different, more appropriate one.[10]

Systemic Illness and Hypogonadism

Obesity, Diabetes Mellitus and Metabolic Syndrome

Data from different studies suggest that testosterone plays an important role in lipid and carbohydrate metabolism and this androgen directly improves insulin sensitivity. Metabolic syndrome is not a disease by itself but a cluster of abnormalities, including visceral type obesity, dyslipidemia, hypertension and impaired glucose metabolism with insulin resistance as the hypothesized underlying pathogenic mechanism.[5]

An association between metabolic syndrome and low testosterone and low SHBG levels is widely accepted. However, the cause and effect relationship is not yet clear. In obesity, increased peripheral conversion of testosterone to estradiol in excess peripheral adipose tissue may lead to suppression of gonadotrophic secretion and subsequent hypogonadism. Another unfavorable effect of obesity may be oxidative stress leading to impaired spermatogenesis.[5] Dyslipidemia also increases oxidative stress. Total testosterone, free testosterone and SHBG are decreased in obese males. Men with low testosterone and low SHBG levels are more likely to develop insulin resistance and diabetes mellitus type 2 (DM 2). On the other hand, insulin is known to inhibit SHBG synthesis; therefore, in insulin-resistant individuals, globulin and, consequently, total testosterone are decreased.

Insulin resistance may be a common factor for both hypogonadism and DM 2. A negative correlation of total testosterone with insulin levels, insulin resistance and BMI in young males with metabolic syndrome was reported.[8] Obesity as well as DM 2 may cause hypogonadism and infertility. The prevalence of hypogonadotrophic hypogonadism among men with DM 2 is increased.

Other Situations Associated with Hypogonadism

Any acute illness such as myocardial infarction, sepsis, severe trauma or stress may lead to secondary (hypogonadotrophic) hypogonadism. Hemochromatosis can affect the hypothalamic-pituitary-testicular axis. Hypogonadism is also observed in liver cirrhosis, chronic renal failure, Crohn's disese, Cushing's syndrome, AIDS, cardiac failure and many other systemic illnesses.

REFERENCES

1. Bay K, Asklund C, Skakkebaek NE, Andersson AM. Testicular dysgenesis syndrome: possible role of endocrine disrupters. Best Pract and Res Clin Endocrinol and Metab 2006;20:77-90.
2. Bhasin S, Cunningham GR, Hayes FJ, Matsumoto AM, Snyder PJ, Swerdloff RS, Montori VM. Testosterone therapy in adult men with androgen deficiency syndromes: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2006;91:1995-2010.
3. Depenbusch M, Nieschlag E. Stimulation of spermatogenesis in hypogonadotropic men. In: Winters SJ (ed) Male hypogonadism: basic, clinical and therapeutic principles. Totowa, NJ: Humana Press, 2004.
4. Greenspan FS, Gardner DG (eds) Basic and Clinical endocrinology, 7th ed. New York etc: McGraw-Hill Companies, 2004.
5. Kasturi SS, Tannir J, Brannigan RE. The metabolic syndrome and male infertility. J Androl 2008; 29:251-59.
6. Kumanov Ph, Nandipati K, Tomova A, Agarwal A. Inhibin B is a better marker of spermatogenesis than other hormones in the evaluation of male factor infertility. Fertil & Steril 2006;86:332-38.
7. Nieschlag E, Swerdloff R, Behre HM, Gooren LJ, Kaufman JM, Legros JJ, Lunenfeld B, Morley JE, Schulman C, Wang C, Weidner W, Wu FCW. Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, and EAU recommendations. Int J Androl 2005;28:125-27.
8. Robeva R, Kirilov G, Tomova A, Kumanov Ph. Low testosterone levels and unimpaired melatonin secretion in young males with metabolic syndrome. Andrologia 2006;38:216-20.
9. Turner HE, Wass JAH. Oxford handbook of endocrinology and diabetes. Oxford: Oxford University Press, 2002.
10. Venetikou MS, Lambou T, Gizani D. Hyperprolactinaemia due to hypothalamic-pituitary disease or drug-induced in patients with erectile dysfunction. Andrologia 2008;40:240-44.
11. Wessells H, Lue TF, McAninch JW. Penile length in the flaccid and erect states: guidelines for penile augmentation. J Urol 1996;156:995-97.

CHAPTER 8

Sexual Dysfunction

Rupin Shah, Vineet Malhotra

INTRODUCTION

The inability to produce an antegrade ejaculation is termed as anejaculation or aspermia. Anejaculation can result from a number of causes and must be treated before any ART procedure can be done.

TYPES

Anejaculation may be *situational or total*: Situational anejaculation is always psychological in origin while total anejaculation may be psychological *(anorgasmic)* or physical *(orgasmic)* in origin.[1]

Situational Anejaculation

In this condition the man is able to ejaculate in some situations (usually at home during intercourse), but has difficulty doing so in other conditions. Typically, situational anejaculation occurs in the clinic (if the man becomes self-conscious), when a man is uncomfortable masturbating, or when a man is under pressure at the time of ovulation. There is no physical problem. Situational anejaculation can usually be treated by vibrator stimulation of the penis, which will produce orgasm and ejaculation in 90% of men.

Anorgasmic Anejaculation (Psychological Anejaculation)

In this condition the man never reaches orgasm (either by masturbation or by intercourse), and hence does not ejaculate. There is no physical problem: the cause is psychological or physiological. Nocturnal emissions are usually present—this is an important indicator that the man is physically normal. Anorgasmic anejaculation can also be treated by vibrator stimulation but with a lower success rate of around 60%. If vibrator therapy fails, electroejaculation can be performed: this will usually succeed in men with situational or anorgasmic anejaculation since there is no physical defect. However, often the sperm quality obtained by electroejaculation is inferior to that obtained by natural ejaculation or by vibrator stimulation.[2] Often, ICSI is required when electroejaculated sperm are used; however, these sperm can be successfully cryopreserved for use in multiple ICSI cycles—fertilization and pregnancy rates with cryopreserved sperm from electroejaculation are at least as good as those obtained with freshly obtained sperm.[3]

Orgasmic Anejaculation (Organic Anejaculation)

The man reaches and experiences orgasm but there is no antegrade ejaculate because of a physical problem that causes failure of emission or retrograde ejaculation. In failure of emission, the sperm is not deposited in to the posterior urethra. This can be because the ejaculatory ducts are blocked by infection (typically, tuberculosis) or because there is damage to the sympathetic nerves and therefore the vas and seminal vesicles do not contract. In men with retrograde ejaculation, spermatozoa are deposited in to the posterior urethra but then flow back in to the bladder because the bladder neck remains open during ejaculation (instead of closing as is normally the case). The bladder neck can become dysfunctional because of surgery, trauma, neuropathy (diabetes) or medicines (alpha-blockers).

Neurogenic failure of emission (e.g. after spinal cord injury, diabetes or lumbar sympathectomy) can be treated by vibratory stimulation or electroejaculation.[4] However, failure of emission due to an anatomical block (e.g. after genitourinary tuberculosis) will need operative sperm retrieval.

Retrograde ejaculation due to diabetic neuropathy often responds to medical treatment with sympathomimetic drugs (ephedrine—25 mg four times a day) in combination with anticholinergics (imipramine—50 mg at night). When the bladder neck has been damaged by surgery or trauma then medical therapy will not help and sperm will have to be retrieved from the bladder after alkalinizing the urine.

SPERM RETRIEVAL TECHNIQUES

Vibrator Therapy

The vibrator works by providing a high intensity stimulus to the penis. This stimulus is strong enough to overcome a psychological or situational inhibition, or a neurogenic defect, and trigger the orgasmic reflex.[5]

The procedure is carried out in a room with complete privacy. Preparatory counseling is important: the procedure is explained and it is emphasized that ejaculation will occur automatically as a result of the vibratory stimulation—the patient should not try and force ejaculation.

The patient passes urine, takes off his clothes and sits on a bed with his legs apart. The vibrator is placed beneath the penis. The penis is placed upon the vibrating head such that the undersurface of the glans and distal shaft are stimulated (Figure 8.1). Once the patient is comfortable with the vibratory sensation, the glans is pressed down upon the vibrator such that the penis receives the maximum amount of stimulation. Keeping the vibrator in place the patient then closes his eyes and fantasizes sexually. Stimulation is continued till ejaculation occurs. This usually occurs in 10 to 30 minutes but some patients with anorgasmic anejaculation, who have never experienced orgasm, may take up to 2 hours of stimulation before they reach orgasm the first time! This period shortens during subsequent sessions. Some patients require a second or third session before they succeed. Others benefit from the use of erotic visual aids (magazines or videos).

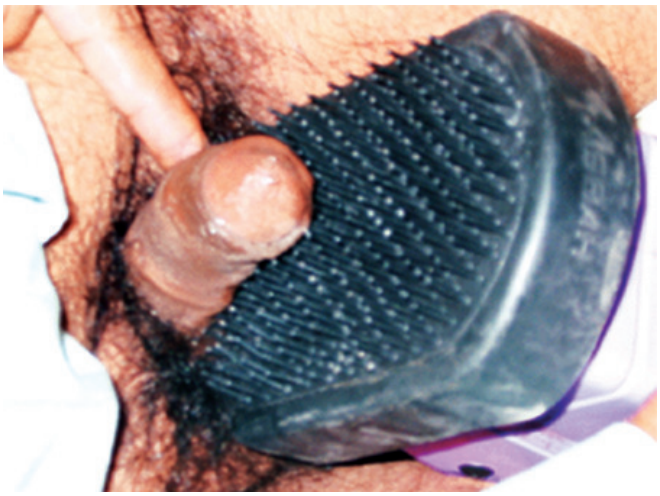


Figure 8.1: Vibrator

Electroejaculation

Electroejaculation involves the direct electrical stimulation of the efferent nerves innervating the seminal vesicles and terminal vas. The most commonly used device is the Seager electroejaculator (Figure 8.2) which delivers a sine-wave, alternating current.[6] The procedure is carried out under general anesthesia (except in paraplegic men with no sensations). The electrodes are mounted on a cylindrical rod that is lubricated and introduced per rectum with the electrodes facing the prostate gland. The voltage is turned up to 5 volts, held for a second and then turned back to 0 volts. For the next stimulus the voltage is increased to 6 volts. The stimulus

Figure 8.2: Electroejaculation system

is progressively increased till ejaculation occurs. Stimulation is then continued at that level till there is no more ejaculation. It is then increased further till ejaculation occurs once more. Usually, the first ejaculation occurs at 5 to 10 volts with a current of 100 to 200 mamps, but some men require a much higher current. If the antegrade ejaculate is scanty, the bladder is catheterized to check for retrograde ejaculation. Since semen parameters are consistent, repeated procedures are not justified for improving the sperm quality in anejaculatory, neurologically intact men;[7] however, men with neurogenic failure (spinal cord injury) may show improvement in semen quality after repeated procedures. If the electroejaculate shows good counts and motility then IUI is done; if the semen quality if poor then it is more costeffective to bypass IUI and proceed directly to IVF-ICSI.[8]

Most men with aspermia can be helped by the use of the vibrator or the electroejaculator. However, if these facilities are not available, or if the man has an anatomical block, then surgical sperm retrieval is required.

Epididymal Sperm Retrieval

Earlier, this was performed as an open microsurgical procedure (MESA). Now this is usually performed percutaneously (PESA). Local anesthesia is given at the root of the scrotum. The head of the epididymis is stabilized between thumb and finger. 0.1 ml of flushing medium is taken in an insulin syringe and the epididymis is punctured directly through the scrotal skin with the 26 G needle. The plunger of the syringe is pulled back to create suction and the needle is moved back and forth within the epididymis, thus aspirating sperm-containing epididymal fluid.

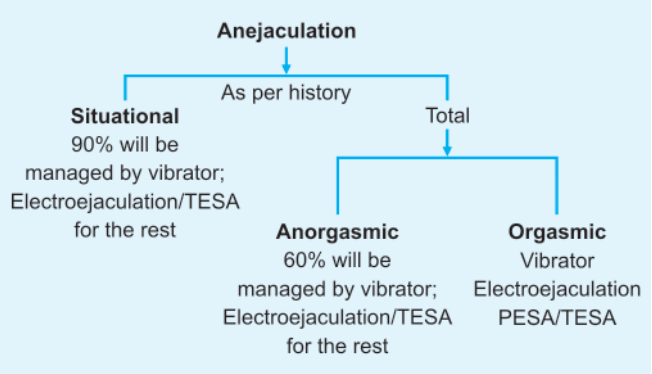
Testicular Sperm Retrieval

In cases of anejaculation, the epididymis is not obstructed, and hence is not turgid. As a result. PESA may not recover sperm. Further, PESA damages the epididymis; hence, in men who have anejaculation, and who have not responded to vibrator or electroejaculation therapy, testicular sperm retrieval (TESA) is often the preferred method of obtaining sperm. An 18 G scalp vein needle is introduced into the testis under local anesthesia. Once the needle enters the tunica, suction is applied using a 10 ml syringe. The needle is pushed in up to its hub, then pulled partly out (staying within the tunica) and then pushed in again. The needle is rotated 180 degrees (to cut the tissue) and the out-and-in motion of the needle is repeated. The tubing of the scalp vein is then clamped and the needle is withdrawn slowly and carefully. As the needle emerges from the scrotal skin, a strand of testicular tissue is pulled out. This is grasped near the skin and pulled out till it snaps or a sufficient length of tubule is obtained. An additional length of testicular tubule will be in the needle and is flushed out gently. The testicular tissue is crushed and the contents are checked for sperm. Additionally, there will be an aspirate within the tubing of the scalp vein and examination of this may also reveal sperm.

Percutaneous vasal sperm aspiration has also been described as a method of obtaining high-motility sperm in men with anejaculation.[9]

The approach to a man who is unable to produce a semen sample is in Flow chart 8.1.

Flow chart 8.1: An approach to ejaculatory failure and management



CONCLUSION

Failure to ejaculate is a common problem and can create a great deal of frustration in the ART laboratory. Situational anejaculation can often be avoiding by proper counseling of the patient. Total anejaculation - both anorgasmic and orgasmic types – need to be diagnosed beforehand through a proper history, so that various remedial measures can be tried out earlier and sperm can be frozen as a backup. The vibrator is an extremely useful and inexpensive device that every ART laboratory should have. The electroejaculator is required less frequently and is expensive; if is not available sperm may be obtained by TESA.

REFERENCES

1. Shah R. Management of anejaculation. In: NP (ed). Handbook of Andrology. Chennai: TR Publishers 1999;129-39.
2. Hovav Y, Shotland Y, Yaffe H, et al. Electroejaculation and assisted fertility in men with psychogenic anejaculation. Fertil Steril 1996;66:620-23.
3. Hovav Y, Yaffe H, Zentner B, et al. The use of ICSI with fresh and cryopreserved electroejaculates from psychogenic anejaculatory men. Human Reprod 2002;17(2):390-92.
4. Nehra A, Werner MA, Bastuba M, et al. Vibratory stimulation and rectal probe electroejaculation as therapy for patients with spinal cord injury: semen parameters and pregnancy rates. J Urol 1996;155:554-59.
5. Shah R. Ejaculatory and erectile dysfunctions in infertile men. In: Jansen R, Mortimer D (Eds). Towards Reproductive Certainty. New York: The Parthenon Publishing Group 1999.
6. Bennett C, Seager S, Vasher E, McGuire E. Sexual dysfunction and electroejaculation in men with spinal cord injury: review. BJU 1991;67:191-94.
7. Hovav Y, Sibirsky O, Pollack RN, et al. Comparison between the first and the second electroejaculate qualities obtained from neurologically intact men suffering from anejaculation. Hum Reprod 2005;20(9):2620-22.
8. Ohl DA, Wolf LJ, Menge AC, Christman GM. Electroejaculation and assisted reproductive technologies in the treatment of anejaculatory infertility. Fertil Steril 2001;76(6):1249-55.
9. Qui Y, Wang SM, Yang DT, et al. Percutaneous vasal sperm aspiration and intrauterine insemination for infertile males with anejaculation. Fertil Steril 2003;79(3):618-20.

CHAPTER 9

Andropause

Vasan SS

INTRODUCTION

Late-onset hypogonadism (LOH)[1] or the earlier term Partial androgen deficiency of the aging male (PADAM or ADAM) is defined as a clinical and biochemical syndrome associated with advancing age, and characterized by a variety of symptoms and a deficiency in serum testosterone levels.[2,48-50] When the term andropause was defined, it created a lot of controversy, because in men, there was no equivalent of menopause. Recent WHO[3] data shows a worldwide total of 400 million people aged 65 and older in the year 2000, a figure estimated to double by 2025 and to eventually reach 2 billion by 2050. Aging is accompanied by a series of signs and symptoms, many of which are rather similar to those observed in young hypogonadal males. The etiology of these signs and symptoms are often multifactorial, making the diagnosis of andropause from clinical signs difficult, which leads to distrust of the symptoms, as well as the diagnostic tools.

Control of Testosterone Production

Testosterone is the major androgen secreted by the testis from its site of production within the Leydig cells. A normal male produces approximately 7 mg testosterone daily but also produces lesser amounts of weaker androgens such as androstenedione and dihydroepiandrosterone. In addition to testosterone, through the actions of the enzyme 5α reductase, dihydrotestosterone is produced by the testis in smaller amounts.

Secretion

Testosterone is secreted at adult levels during 3 epochs of male life - transiently during the first trimester of intrauterine life (coinciding with genital tract differentiation) and again during neonatal life (with unknown physiological significance) and continually after puberty to maintain virilisation. After middle age, circulating total and free testosterone levels decline gradually as gonadotrophin and SHBG levels increase[7-9] with these trends being exaggerated by the coexistence of chronic illness.[8-12] These changes are attributable to impaired hypothalamic regulation of testicular function[13-17] as well as Leydig cell attrition[3] and dysfunction[18,19] so that multiple functional defects are operative throughout the hypothalamopituitary-testicular axis.[20, 21]

Transport

Testosterone circulates in blood at concentrations above its aqueous solubility by binding to circulating plasma proteins. Testosterone binds avidly to sex-hormone binding globulin (SHBG). SHBG is secreted by the liver so its circulating levels are particularly influenced by first-pass effects of oral drugs including sex steroids. Under physiological conditions, 60-70% of circulating testosterone is SHBG-bound with the remainder bound to lower-affinity, high capacity binding sites (albumin, α1-acid glycoprotein, transcortin) and 1-2% remaining non-protein bound. According to the free hormone hypothesis (38-40), the "free" (non-protein bound)

fraction is the most biologically active with the loosely protein-bound testosterone constituting a larger "bioavailable" fraction of circulating testosterone. Nevertheless, "free" and/or "bioavailable" fractions would have enhanced accessibility not only to sites of bioactivity but also sites of inactivation by degradative metabolism. Hence, the net significance of such derived measures of testosterone depends on empirical clinical evaluation, which is very limited.

Circulating testosterone levels demonstrate distinct circhoral and diurnal rhythms. Diurnal patterns of morning peak testosterone levels and nadir levels in afternoon are evident in younger men although this pattern is lost in some ageing men[33] possibly due to increased circulating SHBG levels, reduced testosterone secretion and/or neuroendocrine defects.[16]

Definition

Late-onset hypogonadism (LOH) is a syndrome characterized primarily by:[4] The easily recognized features of diminished sexual desire (libido) and quality and frequency, particularly of nocturnal erections. Other features are changes in mood with concomitant decreases in intellectual ability, congestive functions, spatial orientation ability, fatigue, depressed mood, irritability, sleep disturbances, decrease in lean body mass with associated diminution, increase in visceral fat, decrease in body hair and skin alterations and decreased bone mineral density resulting in osteopenia, osteoporosis and increased risk of bone fractures.

Andropausal Symptoms

These include fatigue 82%, depression 70%, irritability 61%, reduced libido 79%, awareness of premature ageing 43%, aching and stiff joints in the hands and feet 63%, increased sweating especially at night 53%, and classic hot flushes 22%. Last but not least, 80% suffer from erectile dysfunction, reduced early morning erections often being an early warning sign. The age range of 31-80 (mean 54) was wider than that of the menopause in women[45-55] reflecting the importance of the wide range of factors influencing its onset. The overlapping associated factors appeared to be psychosocial stress (59%), alcohol (35%), injuries or operations, particularly vasectomy (32%), medication (31%), smoking (26%), obesity (22%), infections (such as the orchitis caused by mumps and glandular fever, and prostatitis) (11%) and impaired descent of the testes (5%).

Diagnosis

Assessment of Androgen Deficiency – Testosterone Assay

The hormonal picture clearly demonstrated the reasons why this condition remains undiagnosed. The present assay reference ranges of testosterone and bio-available Testosterone provided by clinical laboratories may be an inaccurate way to stratify patients. It leads to confusion in determining which patients are truly "hypogonadal". Further laboratory tests are needed to examine the actual end-organ response to testosterone in LOH with less dependence on traditional parameters. The current problems with measurement of testosterone are:[11]

1. Lack of adequate precision and specificity
2. Availability of different assays with no standardization as regards to assays as well as effect of drugs, proteins, sample tubing, timing of the sample and other constituents on the sample
3. Should we be measuring free testosterone as well as, or instead of, total testosterone? If so, is an androgen index (testosterone/SHBG) as good as free testosterone determination?

The current practices are:

Ruling Out Secondary Hypogonadism

Testosterone is produced by the Leydig cells of the testicles. There is a natural decline in the Leydig cell mass in the aging male with a concomitant decrease in testosterone production. However, secondary hypogonadism may result from hypothalamic- pituitary dysfunction and must be ruled out in the workup of the older male patient. This includes diagnostic tests to identify any concurrent or underlying disease that might be causing the presenting symptoms or low testosterone levels.[22-28] The make-up of the test panel may vary with the ordering physician, but usually includes leutinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, and thyroid stimulating hormone (TSH).

A small percentage of men with low testosterone will have a pituitary abnormality and can be diagnosed with LH and prolactin measurements. Low LH secretion may indicate pituitary dysfunction at the genetic or molecular level, a space-occupying lesion within the sella turcica, or a hyperfunctional pituitary adenoma resulting in hyperprolactinemia (which suppresses LH release via a neuroendocrine pathway).[29-32] The measurement of prolactin is used to rule out prolactinoma, a benign tumor of the pituitary gland that secretes the hormone prolactin.

In some patients, thyroid problems may be the source of andropause-like symptoms. Measurement of thyroid stimulating hormone (TSH) and free T4 will confirm thyroid dysfunction. Hypothyroidism can produce symptoms such as depression, muscular weakness, and fatigue, common symptoms associated with low testosterone.

Testing for Andropause

Diagnosis of andropause is usually based on a combination of clinical impression (rating scales) and laboratory testing.[51]

In men testosterone decreases gradually across a span of several decades. On average, total testosterone concentration decreases from a mean of 600 ng per deciliter (20.8 nmol per liter) at 30 years of age to a mean of 400 ng per deciliter (13.9 nmol per liter) at 80 years, although the range is wide at all ages.[2] By age 80, male hormone levels in many men have dropped to a pre-puberty state.

Testing for andropausal syndrome begins with a test for total serum testosterone administered in the morning (8 to 11 AM) when testosterone is generally at its highest level of the day. Time of day is less of a concern for elderly men, as there is a loss of circadian rhythm with age.

Due to the variability of testosterone levels, it is also recommended to never make the diagnosis based on a single total testosterone level.[53] Total testosterone can be measured by several different laboratory techniques. The most common methods include immunoassays (either direct or extraction-based) and high-performance gas or liquid chromatography (combined with mass spectroscopy).

Immunoassay is the most convenient method for total testosterone measurement. Immunoassays are generally rapid, precise, and easy to use. Fully automated immunoassays have become commonplace in both clinical and research settings. Total testosterone assays measure 3 different circulating forms of the hormone, all in equilibrium. About 40% of circulating testosterone is tightly bound to sex hormone-binding globulin (SHBG). A second form, which is totally unbound or free, represents about 2% of total testosterone. The remaining fraction is loosely bound to albumin and, like free testosterone, is thought to be available for biologic activity. Because the immunoassay technique requires the displacement of testosterone from the binding proteins, the assay's accuracy depends on the completeness of the displacement process. Following displacement, the hormone is captured using an antibody system and quantified.

Although immunoassays are frequently used in many[34-44] hospital laboratories, chromatography is the gold standard for steroid hormone measurement. Gas chromatography coupled with mass spectrometry is the most common method. The downside of this technology is that these methods are complex to perform, time-consuming, and require expensive instrumentation and highly trained technologists.

Confirming Test: Free versus Bioavailable Testosterone

A low level of total testosterone should be confirmed with a repeat analysis. Additional confirmatory tests may include a determination of either free or bioavailable testosterone because total testosterone levels can be a misleading indicator of hypogonadism.

In the aging male, an age-associated increase in SHBG is concurrent with the decline in testosterone, which may result in an even more pronounced decline in the active or bioavailable testosterone fraction. Tests for bioavailable testosterone measure free testosterone and the fraction loosely bound to albumin.

The decision to test for free or bioavailable testosterone depends upon the theoretical approach accepted by the ordering physician. Some physicians believe that only free testosterone is truly biologically active and correlates best with the clinical picture. Others believe that biologically active testosterone includes both free testosterone and testosterone bound to albumin or bioavailable testosterone. Albumin, unlike SHBG, forms only a weak bond with testosterone that is easily broken, making albumin-bound testosterone essentially as biologically active as the free fraction. The 2 most common methods for measuring the free fraction of testosterone are equilibrium dialysis and an immunoassay that uses a labeled testosterone analogue. Unfortunately, neither of these methods is ideal. Although equilibrium dialysis appears to be the most accurate method, it is expensive and time-consuming to perform. Nor is it available in most clinical laboratories, because the testing is complex. Due to the inaccuracy of assays for free testosterone and/or the lack of availability, physicians may order a calculated free testosterone fraction. This calculation is

called the free testosterone index (FTI), using the method of Vermuelen.[52,54] This index uses total testosterone, immunoassayed SHBG, and albumin. There are 2 options for assessing bioavailable testosterone. A direct measurement of bioavailable testosterone may be obtained by performing an ammonium sulfate precipitation to determine the SHBG fraction; and then subtracting the SHBG fraction from the total testosterone result.[55] The free androgen index (FAI) uses the ratio of total testosterone to SHBG multiplied by 100. Which assay correlates best with clinical symptoms is currently under investigation.

In a report comparing the results of various testosterone assays in a cross-sectional study of 50 male subjects age 28 to 90 years, the following assays were studied: total testosterone, free testosterone by equilibrium dialysis, bioavailable testosterone, free testosterone by ultracentrifugation, and direct estimation of serum free testosterone by an analog ligand radioimmunoassay. In addition, 2 calculations were included using total testosterone and SHBG, the free androgen index (FAI) and the free testosterone index (FTI) using the method of Vermeulen.[7] Based on their data, they suggest that the FTI or bioavailable testosterone are the best methods to determine hypogonadism.[56]

Another study found that the calculation for free testosterone correlated better with clinical symptoms than did the analog assay for free testosterone or the ammonium sulfate precipitation method for bioavailable testosterone.[57]

Of course, not all physicians are aware of the methodological issues associated with free or bioavailable testosterone testing. Physicians may be drawing conclusions about testosterone and testosterone testing based on studies that themselves have used some of the less reliable methods. Laboratory professionals must familiarize themselves with the issues surrounding andropause testing. This includes an awareness of the pros and cons surrounding the array of tests and calculations available. Ordering physicians need to understand the caveats attached to any testosterone result reported.

Recommendation:[58] A serum sample should be obtained between 0700 and 1100 hours. The most widely accepted parameters to establish the presence of hypogonadism are the measurement of total testosterone and free testosterone calculated from measured total testosterone and SHBG or measured by a reliable free testosterone dialysis method. Direct radio-immunoassays of free testosterone should not be used.

If testosterone levels are below or at the lower limit of the accepted normal adult male values, it is recommended to perform second determinations together with assessment of serum luteinizing hormone and prolactin. Salivary testosterone has been shown to be a reliable substitute for free testosterone measurements, but cannot be recommended at this time since the methodology has not been standardized and adult male ranges are not available in most hospital or reference laboratories.

Based on the data currently available, the measurement of total blood testosterone is the most appropriate and widely available test to confirm hypogonadism. Subject to future research, a total testosterone level of 300 ng/dl measured in the morning, using a reliable assay,[59] may be used as a threshold below which an individual can be considered hypogonadal.

Questionnaires–Rating Scales

Due to limitations in the assay, a thorough clinical history and physical examination remain important in the decision making algorithm for testosterone replacement therapy. Several questionnaires have been used to collect symptoms or complaints of patients with androgen deficiency (AD), but very few are sufficiently validated. For state of the art validation a scale should be able to: (a) compare relevant symptoms, (b) evaluate the severity of complaints over time, and (c) measure changes pre and post-androgen replacement therapy.

Recommendation: In order to differentiate late on-set hypogonadism or simply aging itself several questionnaires are used and the most popular is the Aging male symptoms scale (AMS). There is an increasing symptom profile, measured by AMS scale, observed among aging men with increasing age, which is independent from medical conditions as well as other confounding factors.

Testosterone Replacement Therapy – General Guidelines to Treatment

Testosterone replacement therapy in aging men is indicated when both clinical findings and signs are suggestive of androgen deficiency, in the form of

decreased testosterone levels are present, provided, that the possible adverse effect of testosterone therapy are evaluated by pretreatment screening. Pretreatment screen should include, medical history for potential sleep apnea, congestive heart failure, symptoms consistent with lower urinary tract obstruction and personal or family history of prostate or breast carcinoma.[34-44] Physical examination, including a digital rectal examination of the prostate. Improvement in signs and symptoms of testosterone deficiency should be sought and failure to benefit in clinical manifestations should result in discontinuation of treatment and further investigation for other causes, is then mandatory.[45]

There is a general agreement[59] that total testosterone level above 12 nmol/L (346 ng/dl), or free testosterone levels above 250 pmol/L (72 pg/mL) do not require substitution. Similarly, based on the data of younger men, there is consensus that serum total testosterone levels below 8 nmol/L (231 ng/dl) or free testosterone below 180 pmol/L (52 pg/ml require substitution. There are no generally accepted lower limbs of normal and it is unclear whether geographically different thresholds depend on ethnic differences or on the physician's perception. Since symptoms of testosterone deficiency become manifest between 12 and 8 mmol/L, trials of treatment can be considered in those in whom alternative cause of these symptoms have been excluded.

Testosterone replacement therapy has been associated with increased hematocrit and hemoglobin, oiliness of skin, and acne. Testosterone therapy needs to be altered or ended if the hematocrit exceeds 52%. Contraindications to therapy include a history of prostate cancer, breast cancer, untreated sleep apnea, and untreated and/or severe congestive heart failure. Exacerbation of sleep apnea and obstructive uropathy related to benign prostatic hyperplasia needs careful monitoring, but is not an absolute contraindication.[46]

Treatment

Current treatment options include oral tablets or capsules, intramuscular preparations, both long and short acting, implantable long acting slow release pellets and transdermal patches, both scrotal and non-scrotal. Neither injectable preparations nor slow release pellets reproduce the circadian pattern of testosterone production of the testes. This is accomplish best by the dermal patches, although oral testosterone may also approximate a circadian rhythm by dose adjustments. Common testosterone preparations are:

INJECTABLES

Testosterone cypionate (Depo-testosterone cypionate) 200-400 mg every 3-4 wks

Testosterone enanthate (Delatestry) 200-400 mg every 4 weeks.

ORAL

Fluoxymesterone (Halotestin) 5-20 mg daily

Methyltestosterone (Metandren) 10-30 mg daily

Testosterone undecanoate(Andriol or Testo-Caps) 120-160 mg daily.

TRANSDERMAL

*Patches:*Testosterone patch (Androderm 6 mg daily or (Testoderm 10/15 mg/day

Creams: Testogel/Andractim

Oral preparations of Testosterone require special consideration. Since most oral testosterone preparations undergo rapid hepatic metabolism they may fail to establish satisfactory serum levels of androgens.[56] Oral agents available include the alkylated (to prevent rapid hepatic metabolism) androgen preparations which generally provide erratic androgenic effects, significant changes in lipid profile, and have a high risk of adverse liver side effects.

Testosterone undecanoate is widely used throughout the world. As a testosterone ester (the only one effective by the oral route), it is free of liver toxicity and effective in bringing levels of serum T within physiological range.

Intramuscular injections of testosterone are usually long acting. These formulations obtain a maximum concentration at approximately 72 hours after injection which slowly diminish over the ensuing 10-14 days. The 17-hydroxyl esters of testosterone most widely used include the short acting testosterone proprionate and the longer acting testosterone enanthate and cypionate.

Effects

These agents have clearly been demonstrated to improve libido, sexual function, potency, energy level, bone density and mood if these abnormalities are caused by androgen deficiency. Supraphysiological levels of serum

testosterone may result in infertility due to suppression of FSH and LH production . As discussed below, there is a justified concern about elevated testosterone levels in elderly men and associated prostate specific antigen (PSA) elevations, increased prostatic size with associated obstructive symptoms and the activation of occult prostatic malignancy.

Transdermal Testosterone Therapy (TTT)

A higher priced but more physiologic approach to testosterone replacement, has recently become available worldwide. TTT is available in both a scrotal and non-scrotal patches. Patches are applied at bedtime with peak testosterone levels achieved in the early morning and a nadir prior to patch replacement. Non-scrotal patches are Androderm and Testoderm systems.

Most common side effects of the dermal patches include the inconvenience of applying them and dermatitis, sometimes leading to significant chemical burns. Androgen Creams, e.g. Testo-Gel, perform even better with less of these dermatoses and better blood levels but cases of skin allergies still occasionally occur.

Other Techniques

Numerous techniques for Testosterone delivery remain investigational. They include long acting implantable percutaneous pellets, spheres, and microcapsules under study in Europe. Because these pellets require the use of a trochar or a minor surgical procedure to be administered, they are less appealing than other Testosterone preparations.

Other Hormones

Growth Hormone/DHEA/Thyroxine/Melatonin, etc. are other hormones often also deficient in aging men and when replaced works synergistically in restoring the symptoms of male andropause. Hence a full Lab Workout has to be conducted to identify these deficiencies and replaced accordingly.

Monitoring to ensure safety, patients should be carefully monitored, particularly in relation to the heart, liver and prostate gland was confirmed by detailed serial tests at periods of three to six months for up to five years.

CONCLUSION

Although many questions about testing for andropause remain, the size and other characteristics of the affected population mean that this will be a growing area in medicine for a long time to come. The media has encouraged the perception that testosterone deficiency may be responsible for the decline in vitality noted by many men who are middle-aged or older. As a result, physicians are receiving more requests for testosterone replacement therapy. The relative benefits and risks of testosterone replacement therapy will continue to be studied. Meanwhile, laboratory professionals should be prepared to not only provide the relevant tests (where practical) but to understand the many issues surrounding various approaches to the testing involved. This will put them in better position to advise physicians on testing options and to place any reported results in the proper context.

REFERENCES

1. Nieschlag E, Swerdloff R, Behre HM, Gooren LJ, Kaufman JM, Legros JJ, Lunenfeld B, Morley JE, Schulman C, Wang C, Weidner W, Wu FC. Investigation, Treatment, and Monitoring of Late-Onset Hypogonadism in Males: ISA, ISSAM, and EAU Recommendations. J Androl. 2006;27(2):135-37.
2. Andropause, Hochreiter WW, Ackermann DK, Brutsch H P . Hirslanden Klinik Aarau, Urologie-Zentrum, Aarau. aarau.hochreiter@uro-hirslanden.ch, Ther Umsch. 2005 Dec; 62(12):821-6. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5206a2.htm.
3. Schubert M, Jockenhovel F. Late-onset hypogonadism in the aging male (LOH): definition, diagnostic and clinical aspects. Klinik II und Poliklinik fur Innere Medizin, Klinikum der Universitat zu Koln, Koln. J Endocrinol Invest 2005;28(3 Suppl):23-7.
4. Bhasin S, Woodhouse L, Storer TW. Androgen effects on body composition. Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Science, 1731 E. 120th Street, Los Angeles, CA 90059, USA. sbhasin@ucla.edu, Growth Horm IGF Res 2003;13 Suppl A:S63-71.
5. Lunenfeld B. Androgen therapy in the aging male. Faculty of Life Sciences, Bar-Ilan University, 52900 Ramat Gan, Israel. blunenf@attglobal.net. World J Urol 2003; 21(5):292-305. Epub 2003 Oct 24.
6. Vinkers DJ, Stek ML, van der Mast RC, de Craen AJ, Le Cessie S, Jolles J, Westendorp RG, Gussekloo J. Generalized atherosclerosis, cognitive decline, and depressive symptoms in old age. Department of Gerontology and Geriatrics, Leiden University Medical Center, C2-R, PO Box 9600, 2300 RC Leiden, The Netherlands. d.j.vinkers@lumc.nl, Neurology. 2005;65(1):107-12.

7. Sohal RS, Mockett RJ, Orr WC. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Department of Molecular Pharmacology and Toxicology, University of Southern California, Los Angeles, CA 90033, USA. sohal@usc.edu, Free Radic Biol Med 2002; 33(5):575-86.
8. Anawalt BD, Merriam GR. Neuroendocrine aging in men. Andropause and somatopause. Department of Medicine, University of Washington School of Medicine, VA Puget Sound Health Care System, Seattle, Washington, USA. Endocrinol Metab Clin North Am 2001; 30(3):647-69.
9. Linja MJ, Visakorpi T. Alterations of androgen receptor in prostate cancer. Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, Biokatu 6, FIN-33520 Tampere, Finland. J Steroid Biochem Mol Biol 2004; 92(4):255-64. Epub 2004 Dec 19.Bottom of Form
10. Goncharov N, Katsya G, Dobracheva A, Nizhnik A, Kolesnikova G, Todua T, Lunenfeld B. Serum testosterone measurement in men: evaluation of modern immunoassay technologies. National Center of Endocrinology, Moscow, Russia. goncharovn@endocrincentr.ru, Aging Male 2005; 8(3-4):194-202.
11. Lunenfeld B, Saad F, Hoesl CE. ISA, ISSAM and EAU recommendations for the investigation, treatment and monitoring of late-onset hypogonadism in males: scientific background and rationale. Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel, Aging Male 2005; 8(2):59-74.
12. Heinemann LA, Saad F, Heinemann K, Thai DM. Can results of the Aging Males' Symptoms (AMS) scale predict those of screening scales for androgen deficiency? Center for Epidemiology and Health Research Berlin, Berlin, Germany. Aging Male. 2004; 7(3):211-18.
13. Morales A, Buvat J, Gooren LJ, Guay AT, Kaufman JM, Tan HM, Torres LO. Endocrine aspects of sexual dysfunction in men. Department of Urology, Queen's University, Kingston, ON, Canada. moralesa@post.queensu.ca, J Sex Med 2004;1(1):69-81.
14. Lue TF, Giuliano F, Montorsi F, Rosen RC, Andersson KE, Althof S, Christ G, Hatzichristou D, Hirsch M, Kimoto Y, Lewis R, McKenna K, MacMahon C, Morales A, Mulcahy J, Padma-Nathan H, Pryor J, de Tejada IS, Shabsigh R, Wagner G. Summary of the recommendations on sexual dysfunctions in men. Department of Urology, UCSF, San Francisco, CA, USA. Tlue@urol.ucsf.edu, J Sex Med 2004;1(1):6-23.
15. Rosen R, Altwein J, Boyle P, Kirby RS, Lukacs B, Meuleman E, O'Leary MP, Puppo P, Robertson C, Giuliano F. Lower urinary tract symptoms and male sexual dysfunction: the multinational survey of the aging male (MSAM-7). Department of Psychiatry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA. rosen@umdnj.edu, Eur Urol. 2003;44(6):637-49.
16. Musicki B, Burnett AL. eNOS function and dysfunction in the penis. Johns Hopkins Hospital, Department of Urology, 600 North Wolfe Street, Baltimore, MD 21287, USA. Exp Biol Med (Maywood). 2006; 231(2):154-65.
17. Miner M, Rosenberg MT, Perelman MA. Treatment of lower urinary tract symptoms in benign prostatic hyperplasia and its impact on sexual function. Brown University, Swansea Family Practice Group, Swansea, Massachusetts 02777, USA.
martin_miner@brown.edu, Clin Ther 2006;28(1):13-25.
18. Rosen RC. Update on the relationship between sexual dysfunction and lower urinary tract symptoms/benign prostatic hyperplasia. Department of Psychiatry, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA. rosen@umdnj.edu, Curr Opin Urol 2006;16(1):11-9.
19. Lowe FC. Treatment of lower urinary tract symptoms suggestive of benign prostatic hyperplasia: sexual function. Department of Urology, St. Luke's-Roosevelt Hospital Center, New York 10019, USA. fclowemd@aol.com, BJU Int. 2005; 95 Suppl 4:12-8.
20. Rosen RC. Update on the relationship between sexual dysfunction and lower urinary tract symptoms/benign prostatic hyperplasia. Department of Psychiatry, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA. rosen@umdnj.edu, Curr Opin Urol. 2006; 16(1):11-9.
21. Burnstein KL. Regulation of androgen receptor levels: Implications for prostate cancer progression and therapy. Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, Florida, USA. kburnste@miami.edu. J Cell Biochem. 2005;95(4):657-69.
22. Dehm SM, Tindall DJ. Molecular regulation of androgen action in prostate cancer. Departments of Urology and Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905. J Cell Biochem 2006 Mar 3.
23. Hoffman MA, DeWolf WC, Morgentaler A. Is low serum free testosterone a marker for high grade prostate cancer? Division of Urology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA. J Urol. 2000;163(3):824-27.
24. Konstantinos H. Prostate cancer in the elderly. 2nd Department of Urology, Aristotle University of Thessaloniki, General Hospital Papageorgiou, 56403, Thessaloniki, Greece. kchatzim@med.auth.gr, Int Urol Nephrol. 2005;37(4):797-806.
25. Barqawi A, Crawford ED. Testosterone replacement therapy and the risk of prostate cancer. Is there a link? Section of Urologic Oncology, University of Colorado Health Sciences Center, Aurora, CO, USA. Int J Impot Res. 2005 Nov 10.
26. Guay AT, Perez JB, Fitaihi WA, Vereb M. Testosterone treatment in hypogonadal men: prostate-specific antigen

level and risk of prostate cancer. Section of Endocrinology and Metabolism, Center for Sexual Function, Lahey Clinic Northshore, Peabody, Massachusetts 01960, USA. Endocr Pract. 2000;6(2):132-8.

27. Ebert T. Clinical experiences with testosterone therapy: prostate safety. Euromed Clinic, Furth, Germany. Aging Male. 2004;7(4):304-11.
28. Tan RS, Salazar JA. Risks of testosterone replacement therapy in ageing men. Baylor College of Medicine and University of Texas Houston, Michael E. DeBakey VA Medical Center, Texas, USA. robert.s.tan@uth.tmc.edu, Expert Opin Drug Saf 2004;3(6):599-606.
29. Gerstenbluth RE, Maniam PN, Corty EW, Seftel AD. Prostate-specific antigen changes in hypogonadal men treated with testosterone replacement. Department of Urology, Case Western Reserve University School of Medicine, University Hospitals of Cleveland, Cleveland Veterans Affairs Medical Center, Ohio 44124, USA J Androl 2002; 23(6):922-26.
30. Jimenez-Cruz JF, Broseta E. Diagnostic challenges of prostatic carcinoma. Service of Urology, La Fe Hospital, Valencia, Spain. Br J Urol 1992;70 Suppl 1:39-42.
31. Vermeulen A, Kaufman JM, Giagulli VA. Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. Department of Endocrinology and Metabolism, University Hospital, Ghent, Belgium. J Clin Endocrinol Metab 1996;81(5):1821-6.
32. Giagulli VA, Kaufman JM, Vermeulen A. Pathogenesis of the decreased androgen levels in obese men. Department of Endocrinology and Metabolism, Medical Clinic, University Hospital, Ghent, Belgium. J Clin Endocrinol Metab 1994;79(4):997-1000.
33. Testosterone Replacement Therapy for Male Aging: ASA Position Statement. J Androl 2006; 27(2):133-34.
34. Shabsigh R, Katz M, Yan G, Makhsida N. Cardiovascular issues in hypogonadism and testosterone therapy. Department of Urology, College of Physicians and Surgeons of Columbia University, New York, New York 10032, USA. rs66@columbia.edu, Am J Cardiol. 2005; 96(12B):67M-72M. Epub 2005 Dec.
35. Kapoor D, Malkin CJ, Channer KS, Jones TH. Androgens, insulin resistance and vascular disease in men. Centre for Diabetes and Endocrinology, Barnsley NHS Foundation Trust Hospital, Barnsley, UK.
36. Berruti A, Tucci M, Terrone C, Gorzegno G, Scarpa RM, Angeli A, Dogliotti L. Background to and management of treatment-related bone loss in prostate cancer. Oncologia Medica, Azienda Ospedaliera San Luigi, Orbassano, Italy. Drugs Aging 2002;19(12):899-910.
37. Isidori AM, Giannetta E, Pozza C, Bonifacio V, Isidori A. Androgens, cardiovascular disease and osteoporosis. Department of Medical Physiopathology, La Sapiens University, Rome, Italy. andrea.isidori@uniroma1.it, J Endocrinol Invest. 2005;28(10 Suppl):73-9.
38. Guillet C, Boirie Y. Insulin resistance: a contributing factor to age-related muscle mass loss? Unite du Metabolisme Proteino-Energetique, UMR Universite d'Auvergne/ INRA, Centre de Recherche en Nutrition Humaine, CHRU de Clermont-Ferrand, France. Diabetes Metab 2005; 31 Spec No 2:20-6.
39. Muhlberg W, Sieber C. Sarcopenia and frailty in geriatric patients: implications for training and prevention. Institut fur Biomedizin des Alterns der Universitat Erlangen-Nurnberg, Medizinische Klinik 2, Klinikum Nurnberg, Prof.-Ernst-Nathan-Str. 1, 90419 Nurnberg, Germany. muehlberg@klinikum-nuernberg.de, Z Gerontol Geriatr. 2004;37(1):2-8.
40. Hogervorst E, Bandelow S, Moffat SD. Increasing testosterone levels and effects on cognitive functions in elderly men and women: a review. Curr Drug Targets CNS Neurol Disord. 2005;4(5):531-40.
41. Lessov-Schlaggar CN, Reed T, Swan GE, Krasnow RE, DeCarli C, Marcus R, Holloway L, Wolf PA, Carmelli D. Association of sex steroid hormones with brain morphology and cognition in healthy elderly men. SRI International, Menlo Park, CA 94025, USA. christina.lessov@sri.com Neurology. 2005;65(10):1591-96.
42. Henkel R, Maass G, Schuppe HC, Jung A, Schubert J, Schill WB. Molecular aspects of declining sperm motility in older men. Department of Urology, Friedrich Schiller University, Jena, Germany. rhenkel@uwc.ac.za, Fertil Steril. 2005; 84(5):1430-37.
43. Meriggiola MC, Pelusi G. Advances in male hormonal contraception. I Clinic of Obstetrics and Gynecology, University of Bologna, Bologna, Italy. cristina.meriggiola@unibo.it, Expert Opin Investig Drugs 2006;15(4):389-97.
44. Testosterone treatment for the aging man: The controversy. Morales A., Queen's University and Centre for Advanced Urological Research, 76 Stuart Street. Kingston, Ontario K7L 2V7, Canada. moralesa@post.queensu.ca, Curr Urol Rep 2004; 5(6):472-77.
45. Darby E, Anawalt BD. Male hypogonadism: An update on diagnosis and treatment. Veterans Affairs Puget Sound Health Care System and University of Washington, Seattle, Washington 98108, USA. bradley.anawalt@med.va.gov, Treat Endocrinol. 2005; 4(5):293-309.
46. Jockenhovel F. Testosterone supplementation: what and how to give. Medical Department, Evangelisches Krankenhaus Herne, Wiescherstrasse 24, 44623 Herne, Germany. Aging Male. 2003; 6(3):200-06.
47. Harman SM, Metter EJ, Tobin JD, et al. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. J Clin Endocrinol Metab 2001;86:724-31.
48. Gray A, Feldman HA, McKinlay JB, et al. Age, disease, and changing sex hormone levels in middle-aged men:

results of the Massachusetts Male Aging Study. J Clin Endocrinol Metab 1991;73:1016-25.

49. Morales A, Heaton JP, Carson CC, 3rd. Andropause: A misnomer for a true clinical entity. J Urol 2000;163:705-12.
50. Morley JE, Charlton E, Patrick P, et al. Validation of a screening questionnaire for androgen deficiency in aging males. Metabolism. 2000;49:1239-42.
51. Summary from the second annual consensus meeting. The Endocrine Society, 2001.
52. Yialamas MA, Hayes FJ. Androgens and the aging male. Endocrinol Rounds 2003;2(1). Available at:

    http://www.endocrinologyrounds.org/. Accessed July 21, 2005.
53. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;84:3666-72.
54. Davies R, Collier C, Raymond M, et al. Indirect measurement of bioavailable testosterone with the Bayer Immuno 1 system. Clin Chem 2002;48:388-90.
55. Morley J, Patrick P, Perry HM. Evaluation of assays available to measure free testosterone. Metabolism 2002;51:554-59.
56. Christ-Crain M, Meier C, Huber P, et al. Comparison of different methods for the measurement of serum testosterone in the aging male. Swiss Med Wkly 2004;134:193-97.
57. Rhoden EL, Morgentaler A. Risks of testosterone-replacement thereapy and recommendations for monitoring. N Engl J Med 2004; 350:482-92.
58. Snyder PJ. Hypogonadism in elderly men — what to do until the evidence comes. N Engl J Med 2004; 350:5.
59. John H, MacIndoe JH. The challenges of testosterone deficiency: Uncovering the problem, evaluating the role of therapy. Postgraduate Med 2003;114:4.

CHAPTER 10

Lifestyle and Environmental Factors Effect on Male Infertility: An Evidence-based Review

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ABSTRACT

Human semen is the end result of a sophisticated biological process hormonally regulated, produced by highly specialized cellular lines that differentiate in embryo but initiate division at puberty and will continue dividing throughout the entire life span of the individual in cycles of 72 days. Semen is a sensitive indicator of environmental, occupational and lifestyle exposures that may be altered by direct toxic effects and hormonal disruption. Damage may happen along the entire human life. However, while some exposures may produce reversible changes, others, especially damage to germinal cells in uterus or prepubertally may result in permanent sequelae. We review the main factors that affect human male fertility and their possible influence in current human reproduction. Some lifestyles, xenoestrogens, heavy metals and volatile organic compounds are already known to compromise reproductive male function. Nonetheless, many questions remain and we still know little about the effect of many other factors on male fertility.

INTRODUCTION

There is mounting evidence that human semen quality and fecundity have been declining during the last decades, at least in large areas of USA and Europe.[1-10] However, those changes may have not taken place homogeneously.[11,12] Geographical variations in semen quality support the idea that local specific factors present in some areas but not in others may be responsible for the decline in semen quality.[13-16] Environmental pollutants, occupational exposures and lifestyle have been explored as possible contributors to those changes.[17,18] Malfunction of the male reproductive system seems to be a good sensitive marker of environmental hazards[19] (Figure 10.1).

In this article we review the current evidence on the association between the main occupational and lifestyles exposures and male infertility.

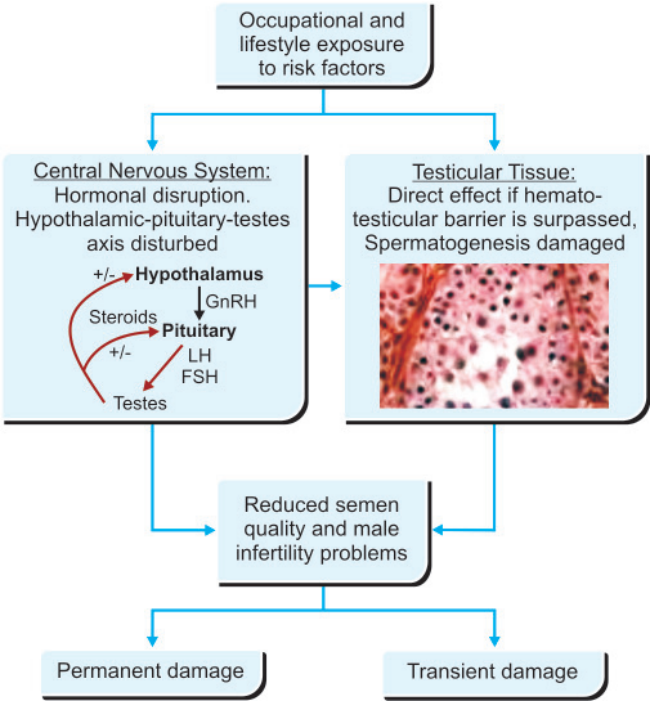

Figure 10.1: Pathways showing the relationship between occupational and lifestyle exposure factors and male infertility injury

LIFESTYLE FACTORS

Special attention has been devoted in the scientific literature to factors that are well established as health risks, like smoking, alcohol and obesity. Other factors that have also being considered in the literature are drug use, genital heat stress, psychological stress and use of cellular phones, although they have received much less attention and there is less conclusive evidence on their impact on semen quality and male fertility.

Smoking

Cigarette smoke is a known somatic carcinogen and cell mutagen. There is also considerable evidence that smoking adversely affect male reproductive health. The impact of cigarette smoking on male fertility has been a highly controversial issue. Some studies did not find association between smoking and sperm quality[20] or sperm DNA damage[21] while others only found effect on sperm volume.[22] However, methodological issues especially the complexity in adjusting for confounding factors may underlie some of these negative findings. Overwhelmingly, it is now clear that smoking has a harmful effect on human male fertility.[23] Tobacco effects can be observed at both, microscopic and molecular levels. Microscopically there is an effect on the sperm concentration, motility and morphology.[24-31] At the molecular level there is increased risk of sperm aneuploidy,[32, 33] higher levels of seminal oxidative stress,[34] alteration of sperm plasma membrane phospholipids asymmetry[35] and sperm DNA fragmentation.[36,37] Furthermore, maternal smoking during pregnancy may have an adverse and irreversible effect on semen quality in male descendants[38] besides a higher risk of birth defects and childhood cancers in the offspring.[39]

Alcohol

Alcoholism has been long associated with reproductive health disturbances such as impotence or testicular atrophy.[40] Spermatogenesis seems to deteriorate progressively with increasing levels of alcohol intake.[41] Chronic alcohol consumption has a detrimental effect on male reproductive hormones and on semen quality.[42] A case-control study conducted in Japan showed that alcohol intake was significantly more common in infertile men than in controls.[43] Alcohol exposures *in vitro* induce reduction of sperm motility and morphology and the response is dose-related.[44] Moreover, there is a higher risk for XY sperm aneuploidy in alcohol drinkers compared to nondrinkers (RR = 1.38; 95% CI: 1.2-1.6).[33] However, we do not know whether all alcoholic beverages have similar adverse effects on semen quality, nor whether there is a safe threshold for alcohol intake. An additional matter of concern is the possible synergistic effect of concurrent toxic habits on male reproduction. A synergistic effect of alcohol and smoking consumption on sperm parameters has been already described, but further research is needed to explore other associations with other lifestyle and occupational or environmental exposures.[45, 46]

Obesity

A common observation in the Western world is the increased average body mass index (BMI) in the general population that has resulted in an increased prevalence of obesity. Several studies have associated lower WHO semen parameters with obesity.[47, 48] In a follow up study in couples enrolled in the Agricultural Health Study in USA, Sallmén et al[49] found, after adjustment for potential confounders, that male BMI was associated with infertility (defined as no pregnancy after 12 months of unprotected intercourse). They found a dose-response relationship between infertility and male BMI and that association was similar for older or younger men. Other authors have found that semen parameters (mainly sperm counts, motility or sperm DNA integrity) and/or reproductive hormones (testosterone, inhibin B, estradiol) are affected in men with BMIs above or below normal levels.[50-55]

Maternal BMI may also have an effect on the future semen parameters of the male offspring, although the issue is far from being elucidated. In a follow-up study Ramlau-Hansen et al[56] found an inverse dose response between maternal BMI and son's Inhibin B hormone. Besides, point estimates for sperm concentration, semen volume, percent motile sperm, testosterone and FSH suggested impaired semen quality in sons of overweight mothers, though the values did not reach statistical significance. The study may not have had enough power to detect real differences and the evidence remains inconclusive.

Recreational Drug Use

There are very few articles exploring the effect of recreational use of cocaine or cannabis, on semen quality

and the male reproductive system, and our knowledge is still very preliminary. In 1990, Bracken et al[57] assessed the association of cocaine use with sperm concentration, motility, and morphology. After adjustment for potential confounders, cocaine use for five or more years was more common in men with low sperm motility, low concentration or large proportion of abnormal forms; while cocaine use within the previous 2 years was twice more frequent in men with oligozoospermia. Authors concluded that given the high prevalence of cocaine use in their male population, the history of cocaine use should be ascertained during diagnostic interviews. Whan et al[58] investigated the effects of delta-9-tetrahydrocannabinol (Delta-9-THC) on human sperm function *in vitro* showing reduced sperm progressive motility and acrosome reaction. Recently, Badawy et al[59] investigated the effects of Delta-9-and Delta-8-THC on sperm mitochondrial O_2 consumption (respiration) showing that this compounds are potent inhibitors of mitochondrial O_2 consumption in human sperm. Overall, these studies emphasize the potential adverse effects of recreational drugs on male fertility although clearly more observational studies are needed.

Genital Heat Stress

Normal sperm production depends on an optimal testicular temperature maintained below body temperature (typically between 34-35°C).[60] Several experimental studies have shown that heat exposures may reduce semen quality.[61-63] In male llamas (*Lama glama*) moderate increases in temperature alters spermatogenesis and all sperm parameters, while showing in histological analysis a higher destruction of tubules and a lower spermatogonial proliferation rate.[64] In humans, occupational activities that require sedentary postures increase scrotal temperature.[65-67] In observational studies it has been found that individuals involved in activities that increase scrotal temperature have poor sperm morphology.[68] Other activities like seating over a heated floor[69] or recreational exposures to wet heat (Jacuzzi or hot baths) also result in impaired semen quality.[70] However, these effects may be reversible once the exposure to heat is ended.

It has also been studied whether the type of underwear used increases scrotal temperature. Jung et al[71] found that scrotal temperature in volunteers wearing wool trousers and shirts fitting to body size were significantly higher for tight versus loose fitting. However, whether that temperature increase results in reduced semen quality remains to be studied. Finally, nocturnal scrotal cooling in infertile men with a history of testicular maldescent and oligozoospermia seems to have a positive effect in improving semen quality after 8 weeks, suggesting that nocturnal scrotal cooling might be a therapeutic option in some patients.[72]

Psychological Stress

The impact of male psychological stress on semen quality is an area of great interest in which further research is needed, especially population based studies. At molecular level, the mechanisms of stress-related semen quality alterations have not been fully elucidated. Eskiocak et al[73] showed that some seminal antioxidant contents (glutathione and free sulfhydryl) as well as motility and morphologically normal spermatozoa decrease in healthy subjects undergoing examination stress. There are a few prospective studies with general population showing that there is a small or nonexistent effect of a man's daily life psychological stress on his semen quality.[74,75] In couples attending fertility clinics, Zorn et al[76] found a weak association between psychological factors and impaired semen quality. In males involved in IVF procedures the quality of the semen sample obtained the day when eggs retrieval was performed was significantly worse than the first sample analyzed in the same patients. The decline in semen quality in the second sample was attributed to the psychological stress involved in that clinical process.[77, 78]

Cellular Telephone Use

There has been an increasing concern about the possibility that the use of cell phones could affect our health and the male reproductive system. A few observational studies have shown that the prolonged use of cell phones may have negative effects on sperm parameters like sperm count, motility, viability, and normal morphology.[79-81] The impact of the radiofrequency electromagnetic waves on semen quality still needs further investigation, including research in animal and *in vitro* models to better understand the mechanisms that are involved in this particular exposure.[82-85]

OCCUPATIONAL AND ENVIRONMENTAL FACTORS

Endocrine Disruptor Compounds (EDCs) like some polychlorinated biphenyls (PCBs),[86-88] organochlorine compounds (pesticides)[89,90] or phthalate esters (PEs),[91]

several heavy metals like lead or cadmium[92-94] and several air pollutants (polycyclic aromatic hydrocarbons [PAHs], dioxins)[95, 96] have been shown to compromise reproductive male function (Table 10.1).

Table 10.1: Major human exposure routes to a number of frequent environmental contaminants

Oral/food exposures	Organochlorine compounds (pesticides) PCBs Phthalates (plasticizers) Heavy metals Dioxins
Air/inhalation exposures	Organochlorine compounds (pesticides) PAHs PCBs Solvents Heavy metals Dioxins
Skin exposures	Phthalates (cosmetics) PCBs

PAHs = polycyclic aromatic hydrocarbons, PCBs = polychlorinated biphenyls.

The alteration of the male reproductive system may result from gonadal endocrine disruption,[97, 98] or by damaging the spermatogenesis process directly.[92,99] Unsurprisingly, occupational activities involving exposures to some of those specific chemicals and toxins are associated with infertility.[93,99-109] But, although there is a growing body of literature relating the effect of specific substances on semen quality, the relationship between environmental chemical exposures and male infertility is not always available. Several studies have explored compared men's semen parameters and occupational exposures in male partners of infertile couples attending fertility clinics.[93, 101, 110-112] In this way an association has been found between welding and reduced semen quality (sperm count and motility).[93, 110] In other case-control studies infertile men had been more frequently exposed to organic solvents,[108,109,112] electromagnetic fields (engineering technicians, etc.) and heavy metals than normozoospermic controls.[93,109,111] Recently, studies have suggested that environmental toxins alter sperm DNA integrity.[113, 114] DNA fragmentation may be an excellent marker of exposure to reproductive toxicants and a diagnostic tool for potential male infertility.[115-117]

Endocrine Disruptor Compounds (EDCs)

EDCs besides causing the "testicular dysgenesis syndrome" (TDS), disturb meiosis in developmental germinal cells.[6, 118] Sharpe and Skakkebaek[118] have suggested that the male reproductive system is most vulnerable to estrogenic agents during the critical period of cell differentiation and organ development in fetal and neonatal life. In this period, the testes are structurally organized, establishing Sertoli cell and spermatogonia numbers to support spermatogenesis that will be initiated at puberty. Endogenous hormones have a vital role in fetal life and to ensure future fertility. The maintenance of tightly regulated estrogen levels is therefore essential for its completion.[97,118] Exposure to the wrong hormones (male fetus exposed to female hormones) or inadequate amounts of these, could affect the reproductive system, by resulting in fertility problems in adulthood.[119, 120] Moreover, due to their chemical composition, EDCs are able to cross a blood-tissue barrier in the testis, suggesting that intratubular germ cells may also be directly exposed.[121, 122]

Dietary soy foods also have estrogenic activity and may affect semen quality. In animal models, genistein crosses the rat placenta and can reach significant levels in fetal brains.[121] In a recent observational study after controlling for potential confounders, Chavarro et al[123] found an inverse association between soy food intake and sperm concentration, that was more pronounced in the high end of the distribution (90th and 75th percentile of intake) and among overweight and obese men.

Another important source of EDCs are pesticides. Juhler et al[124] investigated the hypothesis that farmers with high intake of organic grown commodities would have good semen quality due to their expected lower levels of pesticides exposures. When 40 groups of pesticides were analyzed independently no effect was found on semen quality. However, the analysis did not take into account the synergistic effect that they may have when combined on the reproductive system.[125, 126] A recent work published by the Nordic Cryptorchidism Study Group, studied the human association between maternal exposure to 27 groups of pesticides and cryptorchidism among male children. In a nested case-control study within a prospective birth cohort, researchers compared 62 milk samples from mothers of cryptorchid boys and 68 from mothers of healthy ones and no significant differences were found for any

individual chemical. However, combined statistical analysis of the eight most abundant and persistent pesticides showed that pesticide levels in breast milk were significantly higher in boys with cryptorchidism.[127] Consequently, it is being speculated that male reproductive anomalies (hypospadias, Cryptorchidism)[128] and the global fall in sperm quality[1] might be attributed to the marked increased of EDCs in our water and diet.[129]

In a recent review about the sensitivity of the child to sex steroids and the possible impact of exogenous estrogens, Aksglaede et al[130] concluded that children before puberty are extremely sensitive to estradiol and may respond with increased growth and/or breast development even at serum levels below the current detection limits, and that those changes in hormone levels during fetal and prepubertal development may have severe (probably non reversible) effects in adult life. The authors concluded that, therefore, a cautionary approach should be taken in order to avoid unnecessary exposure of fetuses and children to exogenous sex steroids and endocrine disruptors, even at very low levels. That caution includes food intake, as possible adverse effects on human health may be expected by consumption of meat from hormone-treated animals.[131]

A recent study published by Swan et al[132] suggests that maternal consumption of xenobiotics (anabolic steroids) from beef may damage testicular development in uterus in the offspring and adversely affect reproductive capacity of the males. Sons of "high beef consumers" (>7 beef meals/week) had sperm concentrations 24.3% lower than that of men whose mothers ate less beef.[132, 133] General population is exposed to many potential endocrine disruptors concurrently. Studies, both *in vivo* and *in vitro*, have shown that the action of estrogenic compounds is additive,[134,135] but little is known about the possible synergistic or additive effects of these compounds in humans.[136]

Heavy Metals

Exposure to metals (mainly lead and cadmium) has been long associated with low sperm motility and density, increased morphological anomalies and male infertility.[95,137] Males employed in metal industries had a decreased fertility when compared with other workers as shown by a delayed pregnancy and reduced semen quality.[92, 115, 138-144] Akinloye et al[143] analyzed the serum and seminal plasma concentrations of cadmium (Cd) in 60 infertile males and 40 normozoospermic subjects. Seminal plasma levels of Cd were significantly higher than serum levels in all subjects. A statistically significant inverse correlation was observed between serum Cd levels and all biophysical semen parameters except sperm volume.

Naha et al[144] studied the blood and semen lead level concentration among battery and paint factory workers. Their results included oligozoospermia and increased percentage of sperm DNA haploids, suggesting a diminution of sperm cell production after occupational lead exposure. Additionally, there was a decreased sperm velocity, reduced forward progressive motility with high stationary motile spermatozoa, suggesting retarded sperm activity among the exposed workers. Finally, there is also increased incidence of teratozoospermia associated with high blood and semen lead levels.

In another study that included 98 subjects with light to moderate occupational exposure to lead (Pb) and 51 with no occupational exposure Telisman et al concluded that even moderate exposures to Pb (Blood Pb < 400 µg/L) and cadmium (Cd) (Blood Cd < 10 µg/L) significantly reduced human semen quality without conclusive evidence of a parallel impairment of the male reproductive endocrine function.[146]

Moreover, other reports have also found that blood lead concentration in the general population is negatively correlated with semen quality.[94, 145, 146] Recently Telisman et al[147] reported reproductive toxicity of low-level lead exposure in men with no occupational exposure to metals. In this study, after adjustment, a significant associations was found between blood lead (BPb) and reproductive parameters, such as immature sperm concentration, percentages of pathologic sperm, wide sperm, round and short sperm, serum levels of testosterone and estradiol, and a decrease in seminal plasma zinc and in serum prolactin ($P < 0.05$). These reproductive effects were observed at low-level lead exposures (median BPb 49 µg/L, range 11-149 µg/L in the 240 subjects) that are similar to those of the general population worldwide. However, other articles have been less conclusive in finding adverse effects of lead or cadmium exposure on semen quality or decreased fertility.[148-151] With regards to other possible metals affecting fertility; recently, Meeker et al[152] assessed relationships between environmental exposure to multiple metals (arsenic, cadmium, chromium, copper, lead, manganese, mercury, molybdenum, selenium and

zinc) and human semen quality. The associations involving molybdenum were the most consistent. They found a dose-dependent relationship between molybdenum and declining sperm concentration and morphology in adjusted analysis. These findings are consistent with animal data but more mechanistic studies are needed.

Occupational and Environmental Pollutants

Several solvents may affect human seminal quality[17,107] proportionally to the amount and time of exposure.[108,109] Semen quality in workers exposed occupationally to hydrocarbons like toluene, benzene and xylene present anomalies in viscosity, liquefaction capacity, sperm count, sperm motility, and the proportion of sperm with normal morphology compared with unexposed males.[153-155] An association has also been observed between expositions to styrene in boat building factories workers,[156] PAH in coke-oven workers,[157] and episodic air pollution with an increasing fragmentation of the DNA sperm,[158] as well as altered WHO seminal parameters in young men.[158,159] Dioxin exposure is also associated with impaired male fertility. Recently, Mocarelli et al[96] investigated the reproductive hormones and sperm quality in males that were exposed to the accidental dioxin leak in Seveso, Italy, in 1976. Three groups of males exposed at infancy/prepuberty, puberty, and adulthood, respectively were compared with 184 healthy males. Men exposed in infancy/prepuberty (mean age at exposure: 6.2 years) showed reductions in sperm concentration, progressive motility, total motile sperm count, estradiol and an increase in follicle-stimulating hormone. The other two groups with later exposures (mean age at exposure 13.2 years and 21.5 years of age, respectively) did not have deleterious effects on semen parameters. The study suggests that exposure to dioxins in infancy may permanently reduce semen quality even at relatively low concentrations.

CONCLUSION

There is a growing body of literature showing that a wide variety of substances adversely affects semen quality, and although evidence is not always there, may impair human fertility. However, our knowledge is still fairly limited.

First of all, because although our knowledge is growing on the single effect of individual products, the reality is more complex, there are no single exposures, and there are very few studies addressing the consequences of simultaneous complex exposures to compounds such as food additives, toxicants, contaminants, outdoor and indoor air pollutants, endocrine disruptors and hazardous substances on semen quality and male infertility. A clear side effect of that lack of information is that we may be underestimating the consequences of exposing the population to a wide variety of products because we are missing the larger broader picture of complex exposures.

Secondly, study design does not always facilitate the interpretation of the results. In order to be useful it is necessary to design studies in a way that control of confounding factors is possible, including all known variables that are known to affect semen quality, such as lifestyle, occupational and environmental exposures, and, it would be desirable, along the main developmental stages of the patient's life span.

Finally, in order to better characterize risk assessment, it might be useful to revise the ways to better report damage to sperm quality and to quantity the amount of toxic exposures on similar male reproductive endpoints.

REFERENCES

1. Carlsen E, Giwercman A, Keiding N, et al. Evidence for decreasing quality of semen during the past 50 years. BMJ 1992;305:609-13.
2. Irvine S, Cawood E, Richardson D, et al. Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. BMJ 1996;312:467-71.
3. Swan SH, Elkin EP, Fenster L. Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect 1997; 105:1228-32.
4. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: An analysis of 101 studies published 1934-1996. Environ Health Perspect 2000;108:961-966.
5. Aitken RJ, Koopman P, Lewis SE. Seeds of concern. Nature 2004; 432:48-52.
6. Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod 2001;16:972-8.
7. Skakkebaek NE, Jørgensen N, Main KM, et al. Is human fecundity declining? Int J Androl 2006;29:2-11.
8. Jørgensen N, Asklund C, Carlsen E, et al. Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. Int J Androl 2006;29:54-61.

9. Hauser R. The environment and male fertility: Recent research on emerging chemicals and semen quality. Semin Reprod Med 2006; 24:156-67.
10. Swan SH. Does our environment affect our fertility? Some examples to help reframe the question. Semin Reprod Med 2006;24:142-46.
11. Fisch H, Goluboff ET, Olson JH, et al. Semen analyses in 1, 283 men from the United States over a 25-year period: no decline in quality. Fertil Steril 1996;65:1009-14.
12. Paulsen CA, Berman NG, Wang C. Data from men in greater Seattle area reveals no downward trend in semen quality: Further evidence that deterioration of semen quality is not geographically uniform. Fertil Steril 1996;65:1015-20.
13. Fisch H, Goluboff ET. Geographic variations in sperm counts: A potential cause of bias in studies of semen quality. Fertil Steril 1996; 65:1044-46.
14. Fisch H, Ikeguchi EF, Goluboff ET. Worldwide variations in sperm counts. Urology 1996;48:909-11.
15. Jørgensen N, Andersen AG, Eustache F, et al. Regional differences in semen quality in Europe. Hum Reprod 2001;16:1012-19.
16. Swan SH, Brazil C, Drobnis EZ, et al. Geographic differences in semen quality of fertile US males. Environ Health Perspect 2003; 111:414-20.
17. Tielemans E, Burdorf A, te Velde ER, et al. Occupationally related exposures and reduced semen quality: A case-control study. Fertil Steril 1999;71:690-6.
18. Homan GF, Davies M, Norman R. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatments: a review. Hum Reprod Update 2007;13:209-23.
19. Bonde JP, Giwercman A, Ernst E. Identifying environmental risk to male reproductive function by occupational sperm studies: logistics and design options. Occup Environ Med 1996;53:511-9.
20. Vogt HJ, Heller WD, Borelli S. Sperm quality of healthy smokers, ex-smokers, and never-smokers. Fertil Steril 1986;45:106-10.
21. Sergerie M, Ouhilal S, Bissonnette F, Brodeur J, Bleau G. Lack of association between smoking and DNA fragmentation in the spermatozoa of normal men. Hum Reprod 2000;15:1314-21.
22. Pasqualotto FF, Sobreiro BP, Hallak J, Pasqualotto EB, Lucon AM. Cigarette smoking is related to a decrease in semen volume in a population of fertile men. BJU Int 2006;97:324-6.
23. Vine MF. Smoking and male reproduction: A review. Int J Androl 1996;19:323-37.
24. Vine MF, Tse CK, Hu P, Truong KY. Cigarette smoking and semen quality. Fertil Steril 1996;65:835-42.
25. Zhang JP, Meng QY, Wang Q, Zhang LJ, Mao YL, Sun ZX. Effect of smoking on semen quality of infertile men in Shandong, China. Asian J Androl 2000;2:143-6.
26. Wang SL, Wang XR, Chia SE, Shen HM, Song L, Xing HX, et al. A study on occupational exposure to petrochemicals and smoking on seminal quality. J Androl 2001;22:73-8.
27. Künzle R, Mueller MD, Hänggi W, Birkhäuser MH, Drescher H, Bersinger NA. Semen quality of male smokers and nonsmokers in infertile couples. Fertil Steril 2003;79:287-91.
28. Storgaard L, Bonde JP, Ernst E, Spanô M, Andersen CY, Frydenberg M, et al. Does smoking during pregnancy affect sons' sperm counts? Epidemiology 2003;14:278-86.
29. Jensen TK, Jørgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, et al. Association of in utero exposure to maternal smoking with reduced semen quality and testis size in adulthood: a cross-sectional study of 1,770 young men from the general population in five European countries. Am J Epidemiol 2004;159:49-58.
30. Said TM, Ranga G, Agarwal A. Relationship between semen quality and tobacco chewing in men undergoing infertility evaluation. Fertil Steril 2005;84:649-53.
31. Ramlau-Hansen CH, Thulstrup AM, Aggerholm AS, Jensen MS, Toft G, Bonde JP. Is smoking a risk factor for decreased semen quality? A cross-sectional analysis. Hum Reprod 2007;22:188-96.
32. Shi Q, Ko E, Barclay L, Hoang T, Rademaker A, Martin R. Cigarette smoking and aneuploidy in human sperm. Mol Reprod Dev 2001; 59:417-21.
33. Robbins WA, Elashoff DA, Xun L, Jia J, Li N, Wu G, et al. Effect of lifestyle exposures on sperm aneuploidy. Cytogenet Genome Res 2005;111:371-7.
34. Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ Jr. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil Steril 2002;78:491-9.
35. Belcheva A, Ivanova-Kicheva M, Tzvetkova P, Marinov M. Effects of cigarette smoking on sperm plasma membrane integrity and DNA fragmentation. Int J Androl 2004;27:296-300.
36. Potts RJ, Newbury CJ, Smith G, Notarianni LJ, Jefferies TM. Sperm chromatin damage associated with male smoking. Mutat Res 1999; 423:103-11.
37. Sepaniak S, Forges T, Gerard H, Foliguet B, Bene MC, Monnier-Barbarino P. The influence of cigarette smoking on human sperm quality and DNA fragmentation. Toxicology 2006;223:54-60.
38. Ramlau-Hansen CH, Thulstrup AM, Storgaard L, Toft G, Olsen J, Bonde JP. Is prenatal exposure to tobacco smoking a cause of poor semen quality? A follow-up study. Am J Epidemiol 2007;165:1372-9.
39. Sorahan T, Prior P, Lancashire RJ, Faux SP, Hultén MA, Peck IM, et al. Childhood cancer and parental use of tobacco: Deaths from 1971 to 1976. Br J Cancer 1997; 76:1525-31.
40. Boyden TW, Pamenter RW. Effects of ethanol on the male hypothalamic-pituitary-gonadal axis. Endocr Rev 1983;4:389-95.
41. Pajarinen J, Karhunen PJ, Savolainen V, Lalu K, Penttilä A, Laippala P. Moderate alcohol consumption and disorders of human spermatogenesis. Alcohol Clin Exp Res 1996;20:332-7.

42. Muthusami KR, Chinnaswamy P. Effect of chronic alcoholism on male fertility hormones and semen quality. Fertil Steril 2005; 84:919-24.
43. Tsujimura A, Matsumiya K, Takahashi T, Yamanaka M, Koga M, Miura H, et al. Effect of lifestyle factors on infertility in men. Arch Androl 2004;50:15-7.
44. Donnelly GP, McClure N, Kennedy MS, Lewis SE. Direct effect of alcohol on the motility and morphology of human spermatozoa. Andrologia 1999;31:43-7.
45. Martini AC, Molina RI, Estofán D, Senestrari D, Fiol de Cuneo M, Ruiz RD. Effects of alcohol and cigarette consumption on human seminal quality. Fertil Steril 2004;82:374-7.
46. Kalyani R, Basavaraj PB, Kumar ML. Factors influencing quality of semen: A two year prospective study. Indian J Pathol Microbiol 2007; 50:890-5.
47. Magnusdottir EV, Thorsteinsson T, Thorsteinsdottir S, Heimisdottir M, Olafsdottir K. Persistent organochlorines, sedentary occupation, obesity and human male subfertility. Hum Reprod 2005;20:208-15.
48. Nguyen RH, Wilcox AJ, Skjaerven R, Baird DD. Men's body mass index and infertility. Hum Reprod 2007;22:2488-93.
49. Sallmén M, Sandler DP, Hoppin JA, Blair A, Baird DD. Reduced fertility among overweight and obese men. Epidemiology 2006; 17:520-3.
50. Jensen TK, Andersson AM, Jørgensen N, Andersen AG, Carlsen E, Petersen JH, et al. Body mass index in relation to semen quality and reproductive hormones among 1, 558 Danish men. Fertil Steril 2004; 82:863-70.
51. Koloszár S, Fejes I, Závaczki Z, Daru J, Szöllosi J, Pál A. Effect of body weight on sperm concentration in normozoospermic males. Arch Androl 2005;51:299-304.
52. Fejes I, Koloszár S, Závaczki Z, Daru J, Szöllösi J, Pál A. Effect of body weight on testosterone/estradiol ratio in oligozoospermic patients. Arch Androl. 2006;52:97-102.
53. Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, et al. Impact of body mass index values on sperm quantity and quality. J Androl 2006;27:450-2.
54. Aggerholm AS, Thulstrup AM, Toft G, Ramlau-Hansen CH, Bonde JP. Is overweight a risk factor for reduced semen quality and altered serum sex hormone profile? Fertil Steril 2007; In press.
55. Hammoud AO, Wilde N, Gibson M, Parks A, Carrell DT, Meikle AW. Male obesity and alteration in sperm parameters. Fertil Steril 2008; In press.
56. Ramlau-Hansen CH, Nohr EA, Thulstrup AM, Bonde JP, Storgaard L, Olsen J. Is maternal obesity related to semen quality in the male offspring? A pilot study. Hum Reprod 2007;22:2758-62.
57. Bracken MB, Eskenazi B, Sachse K, McSharry JE, Hellenbrand K, Leo-Summers L. Association of cocaine use with sperm concentration, motility, and morphology. Fertil Steril 1990;53:315-22.
58. Whan LB, West MC, McClure N, Lewis SE. Effects of delta-9-tetrahydrocannabinol, the primary psychoactive cannabinoid in marijuana, on human sperm function in vitro. Fertil Steril 2006; 85:653-60.
59. Badawy ZS, Chohan KR, Whyte DA, Penefsky HS, Brown OM, Souid AK. Cannabinoids inhibit the respiration of human sperm. Fertil Steril 2008; In press.
60. Thonneau P, Bujan L, Multigner L, Mieusset R. Occupational heat exposure and male fertility: a review. Hum Reprod 1998;13:2122-5.
61. Hjollund NH, Storgaard L, Ernst E, Bonde JP, Olsen J. The relation between daily activities and scrotal temperature. Reprod Toxicol 2002;16:209-14.
62. Hjollund NH, Storgaard L, Ernst E, Bonde JP, Olsen J. Impact of diurnal scrotal temperature on semen quality. Reprod Toxicol 2002; 16:215-21.
63. Jung A, Schuppe HC. Influence of genital heat stress on semen quality in humans. Andrologia 2007;39:203-15.
64. Schwalm A, Gauly M, Erhardt G, Bergmann M. Changes in testicular histology and sperm quality in llamas [Lama glama], following exposure to high ambient temperature. Theriogenology 2007; 67:1316-23.
65. Bujan L, Daudin M, Charlet JP, Thonneau P, Mieusset R. Increase in scrotal temperature in car drivers. Hum Reprod 2000;15:1355-7.
66. Sheynkin Y, Jung M, Yoo P, Schulsinger D, Komaroff E. Increase in scrotal temperature in laptop computer users. Hum Reprod 2005; 20:452-5.
67. Jung A, Strauss P, Lindner HJ, Schuppe HC. Influence of heating car seats on scrotal temperature. Fertil Steril 2008;90:335-9.
68. Figà-Talamanca I, Cini C, Varricchio GC, Dondero F, Gandini L, Lenzi A, et al. Effects of prolonged autovehicle driving on male reproduction function: a study among taxi drivers. Am J Ind Med 1996;30:750-8.
69. Song GS, Seo JT. Changes in the scrotal temperature of subjects in a sedentary posture over a heated floor. Int J Androl 2006;29:446-57.
70. Shefi S, Tarapore PE, Walsh TJ, Croughan M, Turek PJ. Wet heat exposure: a potentially reversible cause of low semen quality in infertile men. Int Braz J Urol 2007;33:50-6.
71. Jung A, Leonhardt F, Schill WB, Schuppe HC. Influence of the type of undertrousers and physical activity on scrotal temperature. Hum Reprod 2005;20:1022-7.
72. Jung A, Schill WB, Schuppe HC. Improvement of semen quality by nocturnal scrotal cooling in oligozoospermic men with a history of testicular maldescent. Int J Androl 2005;28:93-8.
73. Eskiocak S, Gozen AS, Yapar SB, Tavas F, Kilic AS, Eskiocak M. Glutathione and free sulfhydryl content of seminal plasma in healthy medical students during and after exam stress. Hum Reprod 2005; 20:2595-600.
74. Hjollund NH, Bonde JP, Henriksen TB, Giwercman A, Olsen J. Reproductive effects of male psychologic stress. Epidemiology 2004; 15:21-7.
75. Fenster L, Katz DF, Wyrobek AJ, Pieper C, Rempel DM, Oman D, et al. Effects of psychological stress on human semen quality. J Androl 1997;18:194-202.

76. Zorn B, Auger J, Velikonja V, Kolbezen M, Meden-Vrtovec H. Psychological factors in male partners of infertile couples: relationship with semen quality and early miscarriage. Int J Androl 2007; In press.
77. Clarke RN, Klock SC, Geoghegan A, Travassos DE. Relationship between psychological stress and semen quality among in vitro fertilization patients. Hum Reprod 1999;14:753-8.
78. Ragni G, Caccamo A. Negative effect of stress of in vitro fertilization program on quality of semen. Acta Eur Fertil 1992;23:21-3.
79. Fejes I, Závaczki Z, Szöllosi J, Koloszár S, Daru J, Kovács L, et al. Is there a relationship between cell phone use and semen quality? Arch Androl 2005;51:385-93.
80. Wdowiak A, Wdowiak L, Wiktor H. Evaluation of the effect of using mobile phones on male fertility. Ann Agric Environ Med 2007; 14:169-72.
81. Agarwal A, Deepinder F, Sharma RK, Ranga G, Li J. Effect of cell phone usage on semen analysis in men attending infertility clinic: An observational study. Fertil Steril 2008;89:124-8.
82. Aitken RJ, Bennetts LE, Sawyer D, Wiklendt AM, King BV. Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. Int J Androl 2005;28:171-9.
83. Erogul O, Oztas E, Yildirim I, Kir T, Aydur E, Komesli G, et al. Effects of electromagnetic radiation from a cellular phone on human sperm motility: an in vitro study. Arch Med Res 2006;37:840-3.
84. Yan JG, Agresti M, Bruce T, Yan YH, Granlund A, Matloub HS. Effects of cellular phone emissions on sperm motility in rats. Fertil Steril 2007;88:957-64.
85. Deepinder F, Makker K, Agarwal A. Cell phones and male infertility: dissecting the relationship. Reprod Biomed Online 2007;15:266-70.
86. Rozati R, Reddy PP, Reddanna P, et al. Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 2002;78:1187-94.
87. Spanò M, Toft G, Hagmar L, et al. Exposure to PCB and p,p´-DDE in European and Inuit populations: impact on human sperm chromatin integrity. Hum Reprod 2005;20:3488-99.
88. Toft G, Rignell-Hydbom A, Tyrkiel E, Shvets M, Giwercman A, Lindh CH, et al. Semen quality and exposure to persistent organochlorine pollutants. Epidemiology 2006;17:450-8.
89. Swan SH. Semen quality in fertile US men in relation to geographical area and pesticide exposure. Int J Androl 2005;29:62-8.
90. Carreño J, Rivas A, Granada A, et al. Exposure of young men to organochlorine pesticides in Southern Spain. Environ Res 2007; 103:55-61.
91. Duty SM, Silva MJ, Barr DB, et al. Phthalate exposure and human semen parameters. Epidemiology 2003;14:269-77.
92. Robins TG, Bornman MS, Ehrlich RI, Cantrell AC, Pienaar E, Vallabh J, et al. Semen quality and fertility of men employed in a South African lead acid battery plant. Am J Ind Med 1997;32:369-76.
93. Irgens A, Krüger K, Ulstein M. The effect of male occupational exposure in infertile couples in Norway. J Occup Environ Med 1999; 41:1116-20.
94. Benoff S, Jacob A, Hurley IR. Male infertility and environmental exposure to lead and cadmium. Hum Reprod Update 2000;6:107-21.
95. Srám RJ, Benes I, Binková B, Dejmek J, Horstman D, Kotˇesovec F, et al. Teplice program—the impact of air pollution on human health. Environ Health Perspect 1996; 104 Suppl 4:699-714.
96. Mocarelli P, Gerthoux PM, Patterson DG Jr, Milani S, Limonta G, Bertona M, et al. Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. Environ Health Perspect 2008;116:70-7.
97. Skakkebæk NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod 2001;16:972-8.
98. Sharpe RM, Irvine DS. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? BMJ 2004;328:447-51.
99. Wyrobek AJ, Schrader SM, Perreault SD, et al. Assessment of reproductive disorders and birth defects in communities near hazardous chemical sites. III Guidelines for field studies of male reproductive disorders. Reprod Toxicol 1997;11:243-59.
100. Alexander BH, Checkoway H, Faustman EM, et al. Contrasting associations of blood and semen lead concentrations with semen quality among lead smelter workers. Am J Ind Med 1998;34:464-69.
101. Bigelow PL, Jarrell J, Young MR, et al. Association of semen quality and occupational factors: comparison of case-control analysis and analysis of continuous variables. Fertil Steril 1998;69:11-8.
102. Viskum S, Rabjerg L, Jorgensen PJ, et al. Improvement in semen quality associated with decreasing occupational lead exposure. Am J Ind Med 1999;35:257-63.
103. Sallmen M, Lindbohm ML, Nurminen M. Paternal exposure to lead and infertility. Epidemiology 2000;11:148-52.
104. Figà-Talamanca I, Traina ME, Urbani E. Occupational exposures to metals, solvents and pesticides: Recent evidence on male reproductive effects and biological markers. Occup Med (Lond) 2001; 51:174-88.
105. Sheiner EK, Sheiner E, Hammel RD, et al. Effect of occupational exposures on male fertility: literature review. Ind Health 2003;41:55-62.
106. Chang HY, Shih TS, Guo YL, et al. Sperm function in workers exposed to N,N-dimethylformamide in the synthetic leather industry. Fertil Steril 2004;81:1589-94.
107. Jensen TK, Bonde JP, Joffe M. The influence of occupational exposure on male reproductive function. Occup Med (Lond) 2006;56:544-53.

108. Cherry N, Moore H, McNamee R, et al. Occupation and male infertility: glycol ethers and other exposures. Occup Environ Med 2008, In press.
109. Mendiola J, Torres-Cantero AM, Moreno-Grau JM, et al. Exposure to environmental toxins in males seeking infertility treatment: a case-controlled study. Reprod Biomed Online 2008;16:842-50.
110. Bonde JP. The risk of male subfecundity attributable to welding of metals. Studies of semen quality, infertility, fertility, adverse pregnancy outcome and childhood malignancy. Int J Androl 1993; 16:1: 1-29.
111. Chia SE, Tay SK. Occupational risk for male infertility: A case-control study of 218 infertile men and 227 fertile men. J Occup Environ Med 2001;43:946-51.
112. Cherry N, Labrèche F, Collins J, et al. Occupational exposure to solvents and male infertility. Occup Environ Med 2001;58:635-40.
113. Stronati A, Manicardi GC, Cecati M, et al. Relationship between sperm DNA fragmentation, sperm apoptotic markers and serum levels of CB-153 and p,p´-DDE in European and Inuit populations. Reproduction 2006;132:949-58.
114. Aitken RJ, De Luiis GN. Origins and consequences of DNA damage in male germ cells. Reprod Biomed Online 2007;14:727-33.
115. Evenson DP, Wixon R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA?). Toxicol Appl Pharmacol 2005;207:(2),532-37.
116. Ozmen B, Caglar GS, Koster F, et al. Relationship between sperm DNA damage, induced acrosome reaction and viability in ICSI patients. Reprod Biomed Online 2007;15:208-14.
117. Meeker JD, Barr DB, Hauser R. Human semen quality and sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides. Hum Reprod 2008;23:1932-40.
118. Sharpe RM, Skakkebaek NE. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993; 341:1392-95.
119. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, et al. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996;104 Suppl 4:741-803. [120]Sharpe RM, Franks S. Environment, lifestyle and infertility- an inter-generational issue. Nat Cell Biol 2002;4:s33-40.
120. Sharpe RM, Franks S. Environment, lifestyle and infertility—an inter-generational issue. Nat Cell Biol 2002;4 Suppl:33-40.
121. Doerge DR, Churchwell MI, Chang HC, et al. Placental transfer of the soy isoflavone genistein following dietary and gavage administration to Sprague Dawley rats. Reprod Toxicol 2001;15:105-10.
122. West MC, Anderson L, McClure N, et al. Dietary oestrogens and male fertility potential. Hum Fertil 2005;8:197-207.
123. Chavarro JE, Toth TL, Sadio SM, Hauser R. Soy food and isoflavone intake in relation to semen quality parameters among men from an infertility clinic. Hum Reprod 2008; In press.
124. Juhler RK, Larsen SB, Meyer O, et al. Human semen quality in relation to dietary pesticide exposure and organic diet. Arch Environ Contam Toxicol 1999;37:415-23.
125. Kortenkamp A. Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. Int J Androl 2008; 31:233-40.
126. Kortenkamp A, Faust M, Scholze M, Backhaus T. Low-level exposure to multiple chemicals: reason for human health concerns? Environ Health Perspect 2007;115 Suppl 1:106-14.
127. Damgaard IN, Skakkebaek, Toppari J, et al. Persistent pesticides in human breast milk and Cryptorchidism. Environ Health Perspect 2006;114:1133-38.
128. Toppari J, Kaleva M, Virtanen HE. Trends in the incidence of cryptorchidism and hypospadias, and methodological limitations of registry-based data. Hum Reprod Update 2001;7:282-86.
129. O´Donnell L, Robertson KM, Jones ME, et al. Estrogens and spermatogenesis. Endocr Rev 2001;22:289-318.
130. Aksglaede L, Juul A, Leffers H, et al. The sensitivity of the child to sex steroids: possible empact of exogenous estrogens. Hum Reprod Update 2006;12:341-9.
131. Andersson AM, Skakkebaek NE. Exposure to exogenous estrogens in food: possible impact on human development and health. Eur J Endocrinol 1999;140:477-85.
132. Swan SH, Liu F, Overstreet JW, et al. Semen quality of fertile US males in relation to their mothers´ beef consumption during pregnancy. Hum Reprod 2007;22:1497-502.
133. vom Saal FS. Could hormone residues be involved? Hum Reprod 2007;22:1503-05.
134. Rajapakse N, Silva E, Kortenkamp A. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ Health Perspect 2002;110:917-21.
135. Tinwell H, Ashby J. Sensitivity of the immature rat uterotrophic assay to mixtures of estrogens. Environ Health Perspect 2004; 112:575-82.
136. Toppari J, Skakkebæk NE. Sexual differentiation and environmental endocrine disrupters. Baillieres Clin Endocrinol Metab 1998;12:143-56.
137. Umeyama T, Ishikawa H, Takeshima H, et al. A comparative study of seminal plasma trace elements in fertile and infertile men. Fertil Steril 1986;46:494-9.
138. Xuezhi J, Youxin L, Yilan W. Studies of lead exposure on reproductive system: A review of work in China. Biomed Environ Sci 1992;5:266-75.
139. Gennart JP, Buchet JP, Roels H, et al. Fertility of male workers exposed to cadmium, lead or manganese. Am J Epidemiol 1992; 135:1208-19.

140. Chia SE, Chan OY, Sam CT, et al. Blood cadmium levels in non-occupationally exposed adult subjects in Singapore. Sci Total Environ 1994;145:119-23.
141. Spinelli A, Figa-Talamanca, Osborn J. Time to pregnancy and occupation in a group of Italian women. Int J Epidemiol 1997;26:601-9.
142. Danadevi K, Rozati R, Reddy PP, et al. Semen quality of Indian welders occupationally exposed to nickel and chromium. Reprod Toxicol 2003;17:451-6.
143. Akinloye O, Arowojolu AO, Shittu OB, et al. Cadmium toxicity: A possible cause of male infertility in Nigeria. Reprod Biol 2006;6:17-30.
144. Naha N, Chowdhury AR. Inorganic lead exposure in battery and paint factory: Effect on human sperm structure and functional activity. J UOEH 2006;28:157-71.
145. Benoff S, Hurley IR, Millan C, et al. Seminal lead concentrations negatively affect outcomes of artificial insemination. Fertil Steril 2003;80:517-25.
146. Telisman S, Cvitkovic P, Jurasovic J, et al. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc and copper in men. Environ Health Perspect 2000; 108:45-53.
147. Telisman S, Colak B, Pizent A, et al. Reproductive toxicity of low-level lead exposure in men. Environ Res 2007;105:256-66.
148. Coste J, Mandereau L, Pessione F, et al. Lead-exposed workman and fertility: A cohort study on 354 subjects. Eur J Epidemiol 1991;7:154-8.
149. Abou-Shakra FR, Ward NI, Everard DM. The role of trace elements in male infertility. Fertil Steril 1989;52:307-10.
150. Hovatta O, Venalainen ER, Kuusimaki L, et al. Aluminium, lead and cadmium concentrations in seminal plasma and spermatozoa, and semen quality in Finnish men. Hum Reprod 1998;13:115-9.
151. Inhorn MC, King L, Nriagu JO, Kobeissi L, Hammoud N, Awwad J, et al. Occupational and environmental exposures to heavy metals: risk factors for male infertility in Lebanon? Reprod Toxicol 2008;25:203-12.
152. Meeker JD, Rossano MG, Protas B, Diamond MP, Puscheck E, Daly D, et al. Cadmium, Lead and Other Metals in Relation to Semen Quality: Human Evidence for Molybdenum as a Male Reproductive Toxicant. Environ Health Perspect 2008;116:1473-9.
153. Xiao G, Pan C, Cai Y, et al. Effect of benzece, toluene, xylene on the semen quality of exposed workers. Chin Med J 1999;112:709-12.
154. De Celis R, Feria-Velasco A, González-Unzaga M, et al. Semen quality of workers occupationally exposed to hydrocarbons. Fertil Steril 2000;73:221-28.
155. De Rosa M, Zarrilli S, Paesano L. et al. Traffic pollutants affect fertility in men. Hum Reprod 2003;18:1055-61.
156. Migliore L, Naccarati A, Zanello A, Scarpato R, Bramanti L, Mariani M. Assessment of sperm DNA integrity in workers exposed to styrene. Hum Reprod 2002;17:2912-8.
157. Hsu PC, Chen IY, Pan CH, Wu KY, Pan MH, Chen JR, et al. Sperm DNA damage correlates with polycyclic aromatic hydrocarbons biomarker in coke-oven workers. Int Arch Occup Environ Health 2006;79:349-56.
158. Rubes J, Selevan SG, Evenson DP, et al. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod 2005;20:2776-86.
159. Selevan SG, Borkovec L, Slott VL, et al. Semen quality and reproductive health of young Czech men exposed to seasonal air pollution. Environ Health Perspect 2000;108:887-94.

SECTION 3

Laboratory Andrology

CHAPTER 11

Semen Analysis

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ABSTRACT

Approximately 15% of the couples trying to conceive are clinically infertile. Male factor is involved in half of these cases. Semen analysis remains the cornerstone of evaluating male infertility. It is one of the first tests done to evaluate a man's fertility. It is inexpensive but can help determine if there is a problem in sperm production or it is the quality of the sperm that is causing infertility. In this chapter, the basics of semen analysis are explained. The tests are simple but can be meaningful if performed by highly trained and specialized professional, in modern laboratories that are accredited to maintain the high standards and have strict quality controls.

INTRODUCTION

Approximately one in six couples is affected by infertility, a problem that can be caused by a number of factors, both male and female (Sigman and Jarow, 2007). The cause is attributed to the female in 30% of cases, to the male in 30%, to both in 30% and is unknown in 10% of cases. The methods for evaluation of male infertility typically have been limited to a semen analysis that evaluates sperm count, motility, and morphology.

A semen analysis evaluates certain characteristics of a man's semen and the sperm contained in the semen. It is an essential component of male infertility investigation, and interpretation of the results plays a vital role in the overall treatment of infertile couples. The basic aim of semen analysis is to evaluate descriptive parameters of the ejaculate (a mixture of spermatozoa suspended along with secretions from the testis, epididymis and other accessory glands).

For a clinician, semen analysis results are predictive of potential fertility and the possible causes of infertility; for an epidemiologist, the results are the basis for assessing hazards in the environment, occupational exposure, or effects of drugs and chemicals. The sample must be obtained and transported to the clinical laboratory according to World Health Organization (WHO) guidelines (WHO, 1999), as semen analysis results can be significantly influenced by both the technique of semen collection and the methods of laboratory analysis.

The WHO manual recommends obtaining two samples for initial evaluation at an interval of not less than 7 days or more than 3 weeks. If the results from the two samples are distinctly different, additional samples have to be collected and examined.

A routine semen analysis includes the following important steps:

PATIENT INSTRUCTIONS FOR SAMPLE COLLECTION, SAFE HANDLING AND DELIVERY

1. Patient should be given clear and simple instructions explaining the need for semen analysis and what is required for specimen collection.
2. Patient should be informed about the importance of abstinence time. Ejaculate must be collected after 3-5 days (but not more than 7 days) of abstinence.
3. Samples should be obtained by masturbation and collected in a warm (20-40°C), sterile, nontoxic plastic or glass wide-mouth container. Prior to sample collection, the patient must void and wash hands and genitals to minimize the chances of contamination.

4. Use of lubricants and saliva should be avoided as their potential toxicity might influence the result. Semen samples should be protected from extremes of temperature (<20°C or >40°C) during transport to the laboratory.
5. All sample containers are labeled with adequate information to eliminate any chances of error.
6. Regular condoms should not be used because of their spermicidal effect.

Ideally, the samples must be collected close to the laboratory. If the specimen cannot be produced close to the laboratory, it must be delivered to the laboratory as soon as possible, certainly within 1 hour of collection. During this period, the sample has to be kept warm by carrying it next to the body, and temperature extremes must be avoided.

INFORMATION ON THE SAMPLE CONTAINER AND PATIENT SHEET

Once the patient has collected the specimen, some preliminary information about the specimen should be obtained:

1. Label the specimen clearly, indicating patient's complete name, clinic number, and collection date.
2. Record collection date and time.
3. Record abstinence time in days.
4. Record time the sample is received at the laboratory.
5. Record information about split ejaculate, noting when the sample was lost and whether the spill occurred at the beginning or after ejaculation.

The samples always should be labeled as "Biohazard" and extreme precaution should be taken. Follow safety guidelines and protocols in handling the sample as the semen sample may contain infectious agents (e.g. hepatitis B, hepatitis C, HIV, herpes simplex).

PHYSICAL EXAMINATION

Macroscopic Examination

Semen Age

Record the time when the sample was received, to the liquefaction time.

Liquefaction

Incubation of the sample must be carried out at either ambient temperature or by placing the specimen in an incubator at 37°C. A normal sample usually liquefies within 60 minutes at room temperature, although usually this occurs within 15-20 minutes. It is determined by the time required for the gelatinous mass to liquefy. A normal sample might contain gel-like gelatinous corpuscles that do not liquefy. Exact liquefaction time is of no diagnostic importance unless >2 hours elapse without any change. This may indicate poor prostatic secretion since the liquefying enzymes are derived from the prostate gland. On the other hand, absence of coagulation may indicate ejaculatory duct obstruction or congenital absence of seminal vesicles.

For samples that do not liquefy, the sample can be mixed by rapidly mixing with a regular transfer pipette or, if the sample is viscous, viscosity can be broken by using a viscosity treatment system.

Color and Odor

It is important to note the color. Normal semen is homogeneously opaque, whitish grey or pearly white. The semen odor is unmistakable and pungent because of sperm oxidation. A yellowish tinge to the semen appears with an increase in the days of abstinence or probably due to carotene pigment. More pronounced yellow discoloration may indicate jaundice or contamination of semen with urine (e.g. bladder neck dysfunction). Drugs like methylene blue and pyridium may also color the semen. Fresh blood (hematospermia) will give semen a reddish tinge, while old blood gives it a brownish tinge. This could be due to the presence of inflammation. Prostatic secretions give semen a strong distinctive odor. Absence or uncharacteristic odor could be associated with an infection.

Volume

Volume of the ejaculate should be measured by transferring the liquefied sample into a graduated 15 mL conical centrifuge tube. The normal volume of ejaculate after 2-5 days of sexual abstinence is about 2-6 mL. Retrograde ejaculation, obstruction of lower urinary tract (urethra, congenital absence of vas deferens, seminal vesicles) may yield low volume. According to the WHO laboratory manual, the reference value for semen volume is ≥ 2.0 mL; however, for clinical purposes; semen volume is differentiated into three categories to facilitate interpretation and diagnosis:

Aspermia: No semen produced after orgasm (seen in certain clinical conditions).

Hypospermia: <0.5 mL of semen ejaculated (partial or complete retrograde flow of semen, accessory glands impairment).

Hyperspermia: > 6 mL of semen ejaculated (long period of sexual abstinence or overproduction of fluids from the accessory sex glands).

If the volume is <1 mL it is important to determine if the sample is complete. The highest sperm concentration is seen in the initial ejaculate.

Viscosity

Viscosity measures the seminal fluid's resistance to flowing. It is measured by the length of the 'thread-lines' or 'spinnbarkeit.' It can be estimated by using a glass rod and observing the length of thread that forms on withdrawal of the rod. A normal sample leaves small, discrete drops; abnormal samples will form threads more than 2 cm long. High viscosity may interfere with determinations of sperm motility, concentration and antibody coating of spermatozoa. Viscosity can be categorized as 'normal', 'moderate' or 'high.' Viscous samples can be treated by a viscosity treatment system containing a premeasured vial of chymotrypsin (5 mg/vial (Conception Technologies, San Diego, CA). The sample can be swirled and left in the incubator for another 10-15 min till viscosity is completely broken down and the sample is suitable for analysis.

pH

The pH of liquefied semen is determined by using pH test strips; pH 6.5 to 10 has been found most suitable for this purpose. A drop of semen is spread evenly onto the pH paper. After 30 seconds, the color of the impregnated zone is compared with the calibrated strip.

Normal semen pH is in the range of 7.2 to 8.2, and it does tend to increase with time after ejaculation. Any change in the normal range of pH may be caused by inflammation of the prostate or seminal vesicles.

MICROSCOPIC EXAMINATION

Wet Preparation Examination

Load a 5 µL of well-mixed semen on a clean, warmed microscope slide with a cover slip on top (18 × 18 mm). If a 22 × 22 mm cover slip is used, the semen volume on the microscope slide should be 10 µL). This preparation has a depth of approximately ~20 µm. A depth less than 20 µm will hamper the rotational movements of the spermatozoa. Care should be taken to avoid formation of air bubbles that can be trapped between the cover slip and the slide. It is important to wait for the drifting to cease/stabilize before examination. In addition, a variety of other disposable two-well 20 µm counting chambers are also available.

A phase contrast microscope is recommended for all examinations of unstained preparations of fresh/washed semen. Initial examination is done under 100× total magnification (10× objective and 10× ocular), which provides an overview for determining mucus strands, sperm aggregation, and evenness of spread of spermatozoa on the slide. Subsequently, the sample should be examined for count and motility under 200× magnification.

Sperm Concentration

Determining accurate sperm concentration (million/mL of ejaculate) and total sperm count (million sperm per ejaculate) is important. The most accurate method of determining sperm concentration is volumetric dilution and hemocytometry. Gently mixing the semen sample using a positive displacement pipette before the volume is withdrawn is essential for an accurate determination of sperm concentration.

Hemocytometry

Principle

A fixed volume of a liquefied semen aliquot is used and fixed sperm are counted in a Neubauer hemocytometer chamber. Dilution of 1:19 is usually employed. Dilutions may be made in small, clean, glass or plastic vials. Extreme care must be taken while making dilutions and preparing the hemocytometer.

Reagents

1. The diluent consists of 50 g sodium bicarbonate
2. 10 ml of 35% of formaldehyde solution, and 0.25 g trypan blue dissolved in reagent water up to 1 liter.

Procedure

1. Filter the solution through Whatman No.1 papers into a clean bottle and store it at 4°C.
2. Add 50 µL liquefied semen to 950 µL diluent. Use a positive displacement pipette to ensure accurate handling of the viscous semen.

3. These dilutions can be stored for up to 4 weeks at 4°C.
4. Place the hemocytometer cover slip over the chamber.
5. Vortex the diluent for 10 s. Transfer 10 µL to each chamber.
6. Leave the hemocytometer in a humid chamber for 10 to 15 minutes for the spermatozoa to settle down onto the counting grid.
7. Count the spermatozoa using a 20× objective phase-contrast optics. The central square of the grid in an improved Neubauer chamber contains 25 large squares, each containing 16 small squares. The number of squares counted depends on the number of spermatozoa seen in the first large square as follows:
   i. < 10 spermatozoa in the first large square - count the whole grid of 25 large squares;.
   ii. 10 - 40 spermatozoa per square - count only 10 large squares (two horizontal or vertical rows); and
   iii. >40 spermatozoa per square - count spermatozoa in the five large squares (the four corners plus the center).

Results

1. Counts of two hemocytometer chambers should be within 5% of their average. If not, discard, remix sample, and prepare another sample to be loaded on the hemocytometer, i.e. (higher value - lower value) must be < (sum of values/20) for the counts to be acceptable
2. Sperm concentration (10^6/mL) = total number of spermatozoa/appropriate correction factor (Table 11.1)
   Total sperm count = Sperm concentration × ejaculate volume.

Table 11.1: Dilution and conversion factors for the improved Neubauer hemocytometer

Spermatozoa per 400X field	*Dilution (semen + diluent)*	*Conversion factors Number of large squares counted*		
<15	1:5 (1 + 4)	20	8	4
15-40	1:10 (1 + 9)	10	4	2
40-200	1:20 (1 + 19)	5	2	1
>200	1: 50 (1 + 49)	2	0.8	0.4

Samples with low numbers of spermatozoa (<2/field, 400× should be centrifuged, a small aliquot of supernatant discarded, and the sample mixed and counted again after correcting for the volume of the supernatant that was removed. Samples in which no spermatozoa are seen must be centrifuged and examined for the presence of spermatozoa in the pellet.

Specialized Counting Chamber

a. Makler chamber

The Makler Counting Chamber (Sefi Medical Instruments, Heifa, Israel) is widely used. It is only 10 microns deep, one-tenth the depth of an ordinary hemocytometer, making it the shallowest of known chambers. It is constructed from two pieces of optically flat glass; the upper layer serves as a cover glass, with a 1 sq. mm fine grid in the center subdivided into 100 squares of 0.1 × 0.1 mm each. Spacing is firmly secured by four quartz pins.

A small number of uncalibrated drops from a well-mixed, undiluted specimen are placed in the center of the chamber by means of a simple rod and immediately covered. A microscopic objective of × 20 is required.

b. Disposable counting chambers

Disposable counting chambers are available with multiple wells and chamber depths. These are available as Micro Cell (Conception Technologies, San Diego, CA) or CellVU (Advanced Meditech International, Flushing, NY). These slides are easy to use. Each slide consists of two separate chambers or wells, each 20 µ in depth.

Loading the Chamber

1. Load an appropriate amount (5 µL) of well-mixed sample to one of the loading zones. The amount of sample will depend on the chamber depth and design. Do not overfill the chamber.
2. The counting chamber fills by capillary action.
3. Wipe away any excess.
4. Counting can be done with an eyepiece reticule consisting of a 10 × 10 box pattern.

Sperm Motility Assessment

Sperm motility is the ratio of the number of motile sperm to total number of sperm in a given volume and is expressed as a percentage. Several scoring systems exist for sperm motility assessments, but a simple grading system is recommended. This provides an assessment of sperm motility without requiring sophisticated equipments.

According to the WHO laboratory manual (WHO, 1999), five microscopic fields are assessed in a systematic way to classify 200 spermatozoa. The motility of each spermatozoon is graded into one of four groups:

a. Rapid progressive motility (i.e. > 25 µm/s at 37 °C and > 20 µm/s at 20°C; note that 25 µm is approximately equal to 5 head lengths or half a tail length).
b. Slow or sluggish progressive motility
c. Non-progressive motility (< 5 µm/s)
d. Immotility

A normal semen analysis must contain at least 50% progressively motile spermatozoa.

Computer-assisted Semen Analysis (CASA)

Manual semen analysis lacks the ability to measure the kinematics of sperm motion. Of the several systems in use for automated semen analysis, computer-aided sperm analysis (CASA) is given much attention because of its potential benefits for analyzing sperm motion (sperm head and flagellar kinematics). Some of these motion characteris-tics have been shown to be related to IVF outcome. Some of the important kinematic parameters are:

i. *Curvilinear velocity:* Curvilinear velocity (VCL) is the measure of the rate of travel of the centroid of the sperm head over a given time period. This is calculated from the sum of the straight lines joining the sequential positions of the sperm along the sperm's track. Values are reported as µm/s.
ii. *Average path velocity:* Average path velocity (VAP) is the velocity along the average path of the spermato-zoon. It is reported as µm/s.
iii. *Straight-line velocity:* Straight-line velocity (VSL) is the linear or progressive velocity of the cell. It is also the straight-line distance between the first and last centroid position for a given period of time. It is reported as µm/s.
iv. *Linearity:* Linearity of forward progression (LIN) is the ratio of VSL to VCL and is expressed as percentage. A value of 100% represents cells swimming in a perfectly straight line.
v. *Amplitude of lateral head displacement:* Amplitude of lateral head displacement (ALH) of the sperm head is calculated from the amplitude of its lateral deviation about the cells axis of progression or average path. It is reported as µm.

A man is considered to be asthenozoospermic if the spermatozoa in his ejaculate show less than 50% forward progressive movement within 60 minutes of ejaculation and necrozoospermic if all sperms are immotile.

Evaluation of Morphology Assessment

For a complete evaluation of a semen sample, the assessment of the morphological characteristics of the spermatozoa is important. The staining of a seminal smear allows the quantitative evaluation of normal and abnormal sperm forms in an ejaculate.

Smear Preparation

Slides should be pre-cleaned with 95% ethanol to allow firm attachment of smears. A small drop of semen, approximately a 5µL aliquot, is placed on the slide. The fraction is then pulled out into a smear with a second slide; this is called the 'feathering' technique. This is done with minimum force to ensure that the spermatozoa tails do not fall apart, and care is taken to guarantee that the smear is not too thick. Two smears are made from each sample. If the sperm concentration is > 20×10^6/ mL, then 5 µL of semen can be used; if the sperm concentration is < 20×10^6/ mL, then 10-20 µL of semen is used. Smears are air-dried and fixed in 95% ethanol for 15 minutes. Air-dried smears can be batched prior to staining.

Staining Methods

The numerous staining techniques available include as the Papanicolaou, Giemsa, Shorr, modified Bryan-Leishman and Diff-Quik methods with Papanicolaou and Diff-Quik being the more common.

Papanicolaou Stain

Papanicolaou stain (WHO, 1999) is the most widely used. It is recommended by the WHO laboratory manual because it gives a good staining to spermatozoa and other cells as it distinguishes basophilic cell components and acidophilic cell components. It allows a comprehensive examination of nuclear chromatin pattern.

Reagents

i. *Fixative:* A freshly prepared solution of equal parts of analytical-grade absolute ethanol and diethyl ether.
ii. Graded ethanol (50%, 70%, 80%, 95%, and 99.5% (v/v)

Staining solutions

i. *Hematoxylin:* Orange G6 and EA-50 are commercially available.
ii. *Acid ethanol:* Prepared by mixing 300 mL of 095% (v/v) ethanol and 2.0 mL concentrated

hydrochloric acid (36% HCL) in 100 mL reagent water.

iii. *Scott's solution:* Prepare by dissolving 3.5 g $NaHCO_3$ and 20.0 g $MgSO_4.7H_2O$ in reagent water to a total volume of 1000 mL.

Procedure

Prepare the air-dried smear and fix as explained above. Proceed with staining according to the sequence.

1.	Graded ethanol (80%, 70%, 50%)	10 dips each
2.	Running water	12-15 dips
3.	Hematoxylin	3 minutes
4.	Running water	3-5 dips
5.	Acid ethanol	2 dips
6.	Scott's solution	4 min
7.	Distilled water	1 dip
8.	Graded ethanol 50%, 70%, 80% and 90%	10 dips
9.	Orange G6	2 min
10.	Ethanol 95%	10 dips
11.	Ethanol 95%	10 dips
12.	EA-50	5 min
13.	Ethanol 95% (3 jars)	5 dips
14.	Ethanol 99.5%	2 min
15.	Xylene (3 staining jars)	1 min

With this stain, the head stains pale blue in the acrosomal region and dark blue in the post-acrosomal region. The midpiece may show some red staining. The tail is stained bluish or reddish and the cytoplasmic droplet stains green.

Diff-Quik Staining

Reagents

Diff-Quik stain (Baxter Healthcare, Deerfield, IL) comprises fixative and two solutions – Diff-Quik I and II.

1. *Diff-Quik fixative*: It contains 1.8 mg/L triarylmethane dye, 100% PDC (pure dye content) in methyl alcohol.
2. *Diff-Quik solution I*: It contains 1g/L xanthene dye 100% PDC, buffer and sodium azide (0.01%) as preservative
3. *Diff-Quik solution II:* It contains 1.25 g/L thiazine dye mixture, 100% PDC (0.625 g/L azure A, and 0.625 g/L methylene blue) and buffer.
4. *Mounting agent*: Accu-mount 60 media.

Procedure

i. Slide is prepared as described above and labeled with the accession number, name, and date.
ii. Proceed with staining: Dip dry slide in Diff-Quik fixative solution containing methanol, 5 times for 1 sec each time and allowing 1 sec between dips.
iii. Allow slide to air dry for 15 min.
iv. Dip dried fixed slide in Diff-Quik solution I (xanthene dye) 3 times for 1 sec each dip and allowing 1 sec between dips. Allow excess stain to drip off. Do not dry slide.
v. Dip slide into Diff-Quik solution II (thiazine dye) 5 times for 1 sec each dip and allow 1 sec between dips. Allow excess stain to drip off. Do not dry slide.
vi. Rinse slide in deionized water gently and thoroughly to remove any excess stain.
vii. Allow stained slide to air dry in drying rack.

Mount cover slip using Accumount on the dried stained slide. For scoring, the slide can be viewed under oil immersion with magnification of 1000 × using a high quality 100 × nonphase-contrast objective and correctly adjusted bright-field optics. About 200 spermatozoa are scored for various abnormalities. The xanthine stain produces the red tones, and the thiazine increases the blue tones.

Scoring Sperm Morphology

Smears can be scored for morphology using the WHO classification (WHO, 1999). Spermatozoa abnormalities are categorized as head, neck and midpiece, and tail defects.

a. *Head defects:* Large, small, tapered, pyriform, round, amorphous, vacuolated (> 20% of the head area occupied by unstained vacuolar areas), heads with small acrosomal area (< 40% of head area), double heads, any combination of these.
b. *Neck and midpiece defects:* Bent neck; asymmetrical insertion of midpiece into head; thick, irregular midpiece; abnormally thin midpiece; any combination of these.
c. *Tail defects:* Short, multiple, hairpin, broken, bent, kinked, coiled tails, or any combination of these.
d. *Cytoplasmic droplets:* Greater than one-third of the area of a normal sperm head.

For a spermatozoon to be normal, the head, neck, midpiece, and tail must be normal. The head should be oval

in shape. The length of the head should be 4.0-5.0 µm and the width 2.5-3.5 µm. The length-to-width ratio should be 1.5-0 to 1.75. Length and width can be measured with an ocular micrometer. The acrosome should be well defined and comprise 40-70% of the head area. The midpiece should be slender, less than 1 µm in width, about one-and-a-half times the length of the head, and attached axially to the head. Cytoplasmic droplets should be less than half the size of the normal head. The tail should be approximately 45 µm long. Reference range is considered as greater than 30% normal forms. In Kruger's strict criteria classification, (Kruger et al. 1986), all 'borderline' forms are considered abnormal. Reference range includes spermatozoa with > 14% normal forms.

Leukocytospermia Test

This test is performed on suspended cells in a liquefied semen specimen and quantitated by counting stained cells in a Makler counting chamber. Peroxidase-positive granulocytes (neutrophils, polynuclear leukocytes, macrophages) are identified by histochemical staining using the Endtz test. This test is often referred to as the myeloperoxidase test.

Preparation of Stock Solution (Stable 6 months)

Reagents

i. Ethanol
ii. Benzidine 0.0625 g
iii. Distilled water 25 mL

Procedure

i. Mix these chemicals in a clean 100 mL bottle. The solution should be clear and yellow.
ii. Cover the bottle with aluminum foil and store in the dark.
iii. Fresh stock solution should be prepared if it gets dark in color or forms a cloudy precipitate.

Benzidine is carcinogenic and should be handled carefully. Wear gloves and a face mask while weighing to avoid accidental contact or inhalation. The expired Endtz test solution should be discarded in concentrated bleach solution.

Preparation of Working Solution

i. Mix 4.0 mL of stock solution and 50 µL of 3% H_2O_2 in a 10 mL tube (dilute 30% stock H_2O_2 10 times).
ii. Cover the bottle with aluminum foil and store in the dark.
iii. Prepare fresh working solution from stock every week and discard old solutions.

Equipment and Materials

1. Tyrode's buffer
2. Makler counting chamber
3. Microcentrifuge tubes
4. Eppendorf pipette and tips (5 µL, 20 µL, 40 µL)

Procedure

i. Measure 20 µL of liquefied semen specimen into a micro-centrifuge tube; add 20 µL of Tyrode's solution and 40 µL of working benzidene solution. Mix and let sit at room temperature for 5 minutes.
ii. Load a Makler counting chamber with 5 µL of the above solution and observe under 10 × magnification.
iii. All granulocytes will stain dark brown in color and retain their round shape.
iv. Count the cells in all the 100 squares of the Makler grid.
v. Number of white blood cells (WBC) can be calculated by multiplying total number of cells by 4 to correct for dilution factor. The total WBC number will be 10^5/mL semen. This number should be corrected to million/ml by dividing by 10.

Report results as million/mL Endtz-positive cells. According to the WHO manual, the normal concentration of WBC in semen is < 1 × 10^6/mL, leukocytospermia is defined as the presence of >1 million/ml WBCs.
Reference range: 0.0-0.9 × 10^6/mL (normal)
Panic value: Endtz test > 1 × 10^6/mL (positive).

Quality Control

A weekly positive control should be run to check reagents. The results should be greater than 1.0 × 10^6/ml Endtz-positive cells.

Note: If semen specimen is not available, an EDTA anti-coagulated blood specimen may be used. Centrifuge the blood specimen to obtain the buffy coat. Remove the buffy layer containing WBC by using a transfer pipette. Dilute into 2 mL of Tyrode's buffer and aliquot (0.1 mL). These aliquots may be used for approximately one month.

Sperm Viability

Sperm vitality is normally measured by testing cellular integrity, assessing the ability of the sperm plasma

membrane to exclude extracellular substances. The cytologically intact 'live' cells can be determined using several vital staining techniques such as eosin Y and trypan blue. The hypo-osmotic swelling (HOS) test is also considered a test of sperm integrity.

Eosin-Nigrosin Stain

An eosin-nigrosin stain must be done on all specimens having a motility of 30% or less. The stain must be performed immediately following the initial motility examination (WHO, 1999).

Reagents

i. *Eosin Y (1%):* Weigh out 0.5 g of eosin Y and add it to 50 mL of deionized water. Dissolve this solution using gentle heat. Cool the liquid to room temperature and filter. This reagent is stable for 3 months at room temperature.
ii. *Nigrosin (10%):* Weigh out 5 g of nigrosin and add it to 50 mL deionized water. Dissolve this solution using gentle heat. Cool the liquid to room temperature and filter. This reagent is stable for 3 months at room temperature.

Procedure

i. Place one drop of well-mixed semen on a Boerner slide.
ii. Add 2 drops of 1% aqueous eosin Y, stir with a wooden stirrer for 15 seconds.
iii. Add 3 drops of 10% aqueous nigrosin. Mix with a wooden stirrer.
iv. Immediately make two thin smears from this mixture by pipetting 10 µL onto each slide and air dry.
v. Place a cover slip with Accu-Mount mounting media (Baxter).
vi. Count 100 sperm on each slide in duplicate using high power (× 40).
vii. Calculate percentage of viable (unstained) and non-viable (stained) sperm.
Viability should be ≥ motility in samples with < 30% motility.

Hypo-osmotic Swelling Test (HOS)

The hypo-osmotic swelling (HOS) test was originally described as a test for sperm function (Jayendran, 1984, WHO, 1999). Now it is most appropriately considered as an additional test for sperm vitality. The HOS test is based on the principle that live spermatozoa withstand moderate hypo-osmotic stress. Dead spermatozoa in which the plasma membrane is no longer intact do not swell, whereas senescent cells show uncontrolled swelling that eventually results in rupture of the overdistended plasma membrane.

Reagents

i. Dissolve 0.735 g sodium citrate dihydrate
ii. 1.351g fructose
iii. Mix in 100 mL of distilled water.

Procedure

i. To 1 mL of HOS solution add 0.1 mL of liquefied semen and mix gently with the pipette.
ii. Incubate at 37°C for 30-60 minutes.
iii. Place a drop of semen mixture on a glass slide and place a cover slip.
iv. Examine under a phase contrast microscope.
v. Observe for tail swelling at × 40 magnification.
vi. Identify number of swollen cells in about 100 spermatozoa in duplicate.

Calculate the mean percentage of swollen cells:

$$\text{Percent swelling} = \frac{\text{Number of sperm with swollen tails}}{\text{Number of spermatozoa with swollen + non - swollen tails}} \times 100$$

Results

Normal values (fertile): > 60% spermatozoa with swollen tails

Abnormal values (infertile): < 50% spermatozoa with swollen tails.

HOS has a limited ability to predict male fertility, but it is useful in selecting non-motile but viable sperm for assisted reproductive technologies. An HOS result < 50% is associated with increased miscarriage rates.

Antisperm Antibodies

Immunological protection to sperm antigens is provided by the tight junctions of Sertoli cells forming the blood-testis barrier. The spermatozoon evokes an immune response when exposed to the systemic immune defense system. In conditions in which this barrier gets disrupted,

formation of antisperm antibodies (ASA) may occur. Systemic and local immunoregulatory mechanisms control the development of antispermatic immunity, which may sometimes be overridden by genetic predispositions, non-physiological routes of inoculation, genital tract infections, etc., which may lead to ASA formation and sperm dysfunction.

Certain ASAs have a cytotoxic effect on the spermatozoa and can cause cell death and immobilization of sperm cells. Other effects of ASAs include creating agglutinated clumps of moving sperm in the semen sample, hampering passage of sperm through the cervical mucus, and zona binding and passage.

Immunobead Test

Principle

Antibodies bound to the human sperm surface can be detected by other antibodies that are against human IgG, IgA or IgM immunoglobulin molecules (Bronson et al 1984; WHO, 1999; Mortimer, 1994a).

Reagents

Immunobeads: Anti-IgG, -IgA and -IgM beads (Irvine Scientific, Santa Ana, CA). For screening, beads for total B-cell labeling can be used. Reconstitute the immunobeads according to the manufacturer's instructions. Beads can be kept for several months at 4°C in the original buffer, which contains a preservative (azide).

Stock buffer: Tyrode's solution or Dulbecco's phosphate-buffered saline (PBS) can be used.

Buffer I (0.3% BSA): Buffer for bead washing (10 mL) and sperm washing (2 x 10 mL for each semen sample). Add 0.6 g bovine serum albumin (BSA; Cohn fraction V) to 200 mL stock buffer. 200 mL buffer is sufficient to wash and run six unknown samples: one positive and one negative control and two sets of IgA and IgG beads.

Buffer II (5% BSA): Buffer for resuspension of beads and sperm pellets, 200 µL for each specimen. Add 250 mg BSA to 5 ml stock buffer. A total of 2 mL buffer II is needed for six samples, two controls and two sets of beads.

1. Filter all solutions through 0.22 or 0.45 µm filters and warm to 25-35°C before use.
2. At least 200 motile sperm should be assessed for each test. A positive (serum from a donor with high titers of antisperm antibodies) and negative control should be included in each run.

Direct Immunobead Test

1. Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate conical centrifuge tubes. Repeat this for each immunobead type.
2. Determine the amount of semen to be used, transfer that volume to a tube and add up to 10 mL with buffer I.
3. Centrifuge all tubes at 500 g for 6 min. at room temperature.
4. *Tubes with sperm:* Discard the supernatants. Resuspend the sperm pellets in 10 ml of fresh buffer I and centrifuge again as above.
5. Discard supernatants and resuspend sperm pellets in 200 µL of buffer II.
6. *Tubes with beads:* Discard the supernatants and resuspend the beads in 200 µL of buffer.
7. Add 5 µL droplets of each immunobead type on clean microscope slides.
8. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip.
9. Place a cover slip on each of the mixtures.
10. Leave the slides for 10 min at room temperature in a moist chamber and then assess under a 20 × phase contrast objective.

Calculations and Results

Only motile sperm should be assessed. Calculate the percentage of sperms that has two or more attached immunobeads. Those that have binding to the tip of the tail should be ignored. Count at least 200 motile sperm in duplicate for every preparation. Record the percentage of sperm carrying attached beads, the Ig class (IgG or IgA) and the site of binding (head, midpiece, and tail).

Indirect Immunobead Test

This is used to detect antisperm antibodies in heat-inactivated seminal plasma.

1. Wash normal donor sperm twice in buffer I as described above (Steps 2-4).
2. Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate conical centrifuge tubes. Repeat this for each immunobead type.

3. Determine the amount of semen to be used, transfer this volume to a tube and add up to 20 mL with buffer I.
4. Centrifuge all tubes at 500 g for 6 min. at room temperature.
5. *Tubes with sperm:* Discard the supernatants. Resuspend the sperm pellets in 10 mL of fresh buffer I and centrifuge again as above.
6. Discard supernatants and resuspend sperm pellets in 200 µL of buffer II or prepare them initially by swim-up procedure or density gradient centrifugation procedure followed by washing.
7. Adjust the washed sperm suspensions to a final motile sperm concentration of 50 × 10^6/mL in buffer II.
8. Dilute 10 µL of the fluid to be tested with 40 µL of buffer II and mix with 50 µL of the washed donor sperm suspension. Incubate at 37°C for 60 min.
9. Wash the sperm twice as described above (Steps 2 – 4).
10. Place 5 µL droplets of each immunobead type on clean microscope slides.
11. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip.
12. Place a cover slip on each of the mixtures.

A positive and negative control should be included in each test run. A positive control can be prepared by using serum from a donor (e.g., from a vasectomized man) with high titers of serum sperm antibodies).

Limitations

Results are based on the analysis of motile sperm. Samples made using sperm with poor motility may give false negative results. A positive finding of > 50% of motile sperm with attached beads is considered to be clinically significant.

Normal Reference Values of Semen Variables

Each laboratory must determine its own reference range for each variable. According to the World Health Organization guidelines (WHO, 1999) the following reference values for the semen sample are suggested:

Reference Value	
Volume	≥ 2.0 mL
pH	≥ 7.2
Sperm concentration	≥ 20 × 10^6 spermatozoa/ mL
Motility	≥ 50% motile (grades a + b) or > 25% with progressive motility (grade a) within 60 minutes of ejaculation
Vitality	> 75% alive
White blood cells	< 1 × 10^6/mL
Immunobead test	< 50% motile spermatozoa with beads bound

SEMEN MICROBIOLOGY AND VIROLOGY

Infection of the male reproductive tract can directly or indirectly cause infertility (Mortimer, 1994b). Inflammation caused by infection or various disorders can affect the secretory function of both the prostate and seminal vesicles. Asymptomatic infections of the prostate can cause partial or complete obstruction of the ejaculatory duct resulting in oligospermia and even azoospermia. Infection of the seminal vesicles often causes substantial reduction in ejaculate volume and a low seminal fructose concentration. Microbiological examination of the semen is required to differentiate a specific microbiological-induced pyospermia from other abnormalities that causes an increase in leukocytes.

Pyospermia

Pyospermia is a laboratory finding categorized as the abnormal presence of leukocytes in human ejaculate. Pyospermia is established when the concentration of seminal WBCs is in the range between 5 × 10^5/mL and 5 × 10^6/mL seminal fluid during semen analysis (Anderson DJ, 1995). Numerous studies have demonstrated that leukocytes in ejaculate have a physiological effect on sperm function, which may further impact male infertility (Wolff H, Anderson DJ, 1988).

The differential diagnosis of symptomatic pyospermia includes infection, autoimmune disease, and inflammation of the accessory sex glands and the lower male urogenital tract. Urogenital infections include acute and chronic prostatitis, seminal vesiculitis, epididymo-orchitis, cystitis, urethritis, urethral stricture, stone disease, foreign bodies, upper urinary tract infection, retrograde ejaculation, and localized sepsis of the adjacent lower gastrointestinal tract and asymptomatic bacteriuria. Chronic infections that may result in pyospermia include fungal, mycobacterial, and congenital lesions that cause urogenital tract infection.

Collection of Semen Specimens

1. Hands must be washed thoroughly with antiseptic soap.
2. Penis should be washed using antiseptic solution.
3. The semen specimen should be collected by masturbating directly into the sterile container.

Organisms Found in Semen

Many organisms found in semen are actually contaminants from the patient's skin or from the air at the time of collection. Not all are associated with pyospermia, hence caution must be exercised in interpretation of positive cultures.

Ureaplasma and Mycoplasma

Ureaplasma and mycoplasma species are common commensal inhabitants of the lower genitourinary tract in adolescents and adult men and women who are sexually active. The organisms can be transmitted venereally and vertically from mother to offspring. *Ureaplasma urealyticum* and to a lesser extent *Mycoplasma hominis* are therefore often found in semen of infertile patients. *U. urealyticum* is part of the normal genital flora of both men and women and is found in about 70% of sexually active humans. Their presence is abnormal, and most authorities agree that the infection must be treated with antibiotics.

Chlamydia trachomatis

Chlamydia trachomatis is occasionally present in human semen. It is the cause of the most prevalent sexually transmitted bacterial disease worldwide and is responsible for an estimated 90 million infections. Considering the high worldwide prevalence of *C. trachomatis* infection, artificial insemination by donor (AID) is a potential route for the spread of *C. trachomatis* and has been reported as such. It cannot be cultured, since seminal plasma factor is toxic to the cell lines used for the culture. Treatment must extend to both the partners.

Neisseria gonorrhoeae

This organism causes severe symptoms in men. It can be identified in semen as gram-negative intracellular diplococci. Transmission is through sexual intercourse as well.

Trachomonas vaginalis and E. coli

In men with urinary tract infections, *E.coli* can colonize the prostate and cause production of IgA leading to sperm agglutination. Other organisms that form the normal flora of the reproductive tract may commonly be present; these include enterococci such as *S. faecalis* and staphylococci such as *S. epidermidis. Mycobacterium tuberculosis* maybe present in the absence of pyospermia.

Viruses

Many viruses can be isolated from semen, but the most important is human immunodeficiency virus (HIV). Its presence can be detected by either culture or polymerase chain reaction. Other viruses that could be present in semen are human T-cell lymphotrophic virus type I (HTLV-1), hepatitis B, hepatitis C, cytomegalovirus (CMV), and human papilloma virus (HPV). Genital herpes virus often is isolated from semen in infected patients.

BIOCHEMICAL ASSESSMENT OF SEMINAL PLASMA, PROSTATE, EPIDIDYMIS AND SEMINAL VESICLES

The prostate, seminal vesicles, and epididymis produce components such as zinc, citric acid and α-glucosidase that are uniquely specific to each accessory gland.

Measurement of Zinc

A colorimetric assay kit is used for determining the zinc content in the seminal plasma (Johnson and Eliasson, 1987, Mortimer, 1994c). It can be done in either a -well plate or spectrophotometer cuvettes. The assay is based on the principle that in the presence of zinc, 5-Br-PAPS (2-(5 bromo-2 - pyridylazo)-5-(N-propyl-N-sulphopropylamino)-phenol is converted to 5-Br-PAPS-Zn complex, which absorbs at 560 nm.

Reagents

1. *Zinc kit:* Chromogen solution is prepared by mixing color reagents A and B in proportions of 4:1. It is stable for 1 week at 4°C.
2. *Zinc standard:* (0.1 mM).

Specimens

1. Centrifuge semen at 1000 g for 15 minutes. 100 µL of semen is centrifuged to obtain 10 µL of neat seminal plasma.

2. A 10 µL aliquot of cell-free seminal plasma is diluted with 600 µL of water.

Procedure

1. Set absorbance readings on the spectrophotometer to 560 nm, and allow adequate time for stabilization.
2. Prepare a standard curve in duplicate (100 µM diluted to give 80, 60, 40, 20 and 10 µM).
3. Set the spectrophotometer to zero with a cuvette containing reagent water.
4. Add 2.5 mL working chromogen solution (mix 4 parts of color reagent A with 1 part of color reagent B). Add 20 µL of color reagent to 40 µL of diluted semen samples, standard, and blank.
5. Leave at room temperature for 5 minutes.
6. Measure absorbance at 560 nm and calculate results.
7. Calculation: Multiply by a dilution factor to obtain the concentration of zinc (mM) in undiluted seminal plasma. Multiply by ejaculate volume to obtain µmol/ejaculate.

Results

Zinc is a specific marker of prostatic function. Normal range of seminal zinc is 1.2-3.8 mmol/L or ≥ 2.4 µmol per ejaculate.

Measurement of Citric Acid in Seminal Plasma

Citric acid is an indicator of prostatic gland function. Decreased citric acid levels may indicate either prostate dysfunction or prostatic duct obstruction. It can be measured using the Boehringer enzymatic, NADH – linked kit (Mortimer, 1994c).

Reagents

1. *Boehringer Kit No. 130976:* Contains Solution 1: 3 × Bottle1 (mainly NADH), which is reconstituted by adding 12 mL reagent water and shaking well. 3 × Bottle 2 (citrate-lyase), reconstituted by adding 0.3 mL reagent water and shaking well.
2. *Triethanolamine buffer (pH 7.7):* Prepared by dissolving 14.9g triethanolamine in 750 mL reagent water and adjusting the pH to 7.6 by adding 1 N HCl. Dissolve 0.027 g $ZnCl_2$ in 250 mL reagent water and add it to the triethanolamine solution. Add 0.5 g of sodium azide and mix thoroughly.
3. *Trichloroacetic acid (TCA, 15%):* Dissolve 15 g trichloroacetic acid in 100 mL reagent water).
4. *NaOH (6 N):* Dissolve 24 g NaOH pellets dissolved in 100 mL reagent water.
5. *Citric acid standard:* 0.174 g citric acid in 10 mL reagent water. Make a 1 + 57 dilution.

Specimens

1. Centrifuge 250 µL liquefied semen (cell- and protein-free) in an Eppendrof tube at 1000 g for 15 minutes.
2. Add 100 µL of supernatant to 4.95 mL of 15% TCA in a small, capped vial and shake well.
3. Add 0.75 mL NaOH (6 N) and adjust the pH to 7.0.
4. Freeze three 0.5 mL aliquots of the extract in Eppendorf tubes at – 20°C.

Procedure

1. Set the spectrophotometer to 340 nm and allow adequate time for stabilization.
2. Mix 0.5 mL of solution 1, 2.3 mL TRA buffer and 0.2 mL sample, standard, or blank in a disposable cuvette. Prepare each set in duplicate.
3. Adjust the spectrophotometer reading to zero with a cuvette containing reagent water.
4. Measure initial absorbance (A1).
5. Add 20 µL of solution 2. Shake it well, wait exactly 5 minutes, and measure the absorbance again (A2).
6. Calculate and analyze the results according to the formula:

   $\Delta A \times (V \div V) \times DF \times MW \div E \div D + 100 = g/L$ where

   DF = Specimen dilution factor

   V = final volume (3.02 mL)

   MW = molecular weight of the substance analyzed (192.1)

   E = absorption coefficient of NADH at 340 nm (6.3 $cm^3/\mu mol$)

   D = light path (1 cm)

   V = sample volume (0.2 mL)

   $\Delta A \times 139.0$ = mmol citric acid/ L.

Results

The normal range of seminal plasma citric acid concentration is 9.4-43.4 mmol/L or ≥ 52 µmol per ejaculate. Decreased levels of citric acid may indicate either prostatic dysfunction or duct obstruction. Further evaluation must be done by physician.

Measurement of Neutral α-glucosidase in Seminal Plasma

Seminal plasma contains both neutral α-glucosidase isoenzyme that originated from the epididymis and an acid isoenzyme contributed by the prostate. The latter can be selectively inhibited to allow measurement of neutral α-glucosidase. P-nitrophenol α-glucopyranoside in the presence of α- glucosidase is converted to p-nitrophenol, and the absorbance can be read at 405 nm (Cooper et al 1990, Mortimer, 1994c).

Reagents

1. Phosphate buffer (0.2 M, pH 6.8). Prepare 1% SDS in phosphate buffer.
2. Color reagent 1 for stopping the reaction. 0.1 M sodium bicarbonate.
3. Color reagent 2 for diluting the product. Prepare color reagent 1 containing 0.1% SDS.
4. Substrate (p-nitrophenol glucopyranoside (PNPG, 5mg/mL in phosphate buffer, pH 6.8). Prepared fresh.
5. Glucosidase inhibitor for semen blanks Castanospermine (10 mM). Prepare 1mM working solution. Freeze in aliquots at – 20ºC.
6. 100 mM solution of sodium carbonate.
7. Standard: 5 mM p-nitrophenol. Make fresh every time.

Specimen

Use sperm-free seminal plasma prepared by centrifuging an aliquot of semen at 1000 g for 15 minutes.

Procedure

1. Set a water bath at exactly 37°C for the incubation step below.
2. Thaw specimens to be assayed and mix well.
3. Prepare 100 µL of PNPG substrate solution in Eppendrof tube.
4. Using a positive displacement pipette, add 10 µL specimen aliquots in duplicate into the Eppendorf tube.
5. Mix each tube and incubate at 37°C for 2 h.
6. Include internal quality control samples consisting of high, medium, and low activities of neutral α-glucosidase.
7. To two high activity quality control semen pools. Add 8 mL of 1 mM castanospermine to provide semen blank value.
8. Prepare PNP standard curve (160, 120, 80, 40 µm) with color reagent 2 (within an hour of incubation).
9. Stop the reaction by adding 1.0 mL of color reagent 1 and mix.
10. Read absorbance of each sample at 405 nm against the blank (water).

Results

1 unit of glucosidase activity is equal to the production of 1 µ mole product (PNP) per minute at 37ºC. In this assay, the activity is derived from 15 µL of semen in a total volume of 1.115 mL over 120 minutes. Therefore the correction factor is 1115/15/120 or 0.6194.

1. Read the concentration of PNP produced by the sample from the standard curve (µM).
2. Multiply by the correction factor (0.6194) to obtain the activity of neutral glucosidase in undiluted seminal plasma (U/l).
3. Subtract the activity of the castanospermine semen blank from each sample to obtain the corrected (glucosidase-related) activity.
4. Multiply the corrected activity by the ejaculate volume to obtain glucosidase activity (mU) per ejaculate. α-glucosidase is a specific indicator for epipdymis function. Normal values are ≥ 20 mU/ ejaculate.

Quantitative Seminal Fructose

Sperm in semen sample are lysed, and addition of resorcinol and subsequent heating at 70ºC results in a salmon-pink color, which is read at 420 nm (Davis and Gander, 1967, Moon and Bunge, 1968).

Reagents used

1. Concentrated HCl
2. Deionized water.
3. Fructose (0.32 mmole/L). Add 14.4 g of fructose to make 250 mL of deionized water (5.56 mg/dL).
4. Resorcinol 0.05%. Add 25 mg of resorcinol to 50 mL of ethanol (95%).

Freeze three aliquots (150 µL) aliquots of supernatant in 1.5 mL Eppendorf tubes at – 20°C.

Procedure

1. Turn on the 77ºC water bath.
2. Set spectrophotometer absorbance at 420 nm.
3. Adjust the reading to zero with cuvette containing reagent water.

4. Label three beakers for patient, positive control (pooled seminal plasma from normal donors), and negative controls (no semen added).
5. *Treat with acid:* To a clean beaker add 7.5 mL of deionized water + 2.5 mL HCl and 50 µL of semen or seminal plasma. Mix each patient and control sample carefully (200-fold dilution).
6. Label beaker for each patient and control. Using a Whatman # 1 filter paper, filter each patient and control mixture into appropriately labeled beaker.
7. Label in duplicate 13 × 100 mm glass tubes for each standard, control, and patient sample. Add the following to the appropriate tube (Table 11.2).
8. Mix the tubes carefully and add 3.0 mL of concentrated HCL to each tube under the fume hood. Mix carefully.
9. Add 1.90 mL of 0.05% resorcinol to each tube, cap and vortex carefully.
   Incubate all tubes at 77°C for 8 min.
10. Place the tubes in an ice bath. Cool to room temperature. Transfer to disposable cuvettes and read the tubes at 420 nm.
11. Calculate the average OD from the standard. Multiply by the dilution (200) to obtain the final results in mg/dL.

Results

Fructose is a marker for seminal vesicle function. Normal range for seminal fructose is >150 mg/dl.

SAFETY AND QUALITY CONTROL IN THE ANDROLOGY LABORATORY

All general safety precautions related to electrical or chemical hazards, compressed gases, fire, and physical injury must be observed in the laboratory. Safety precautions must be exercised when handling biological specimens in the laboratory. All semen samples must be treated as a biological hazard, and extreme caution should be exercised. This includes use of protective clothing, eye protection, use of safety gloves, and any other protective measures as necessary.

Table 11.2: Preparing a sample for fructose measurement

Fructose standard (mg/dL)	*Fructose stock reagent (mL)*	*Deionized water (mL)*	*Patient sample or control (mL)*
0	0	1.0	
0.36	0.63	0.945	
0.72	0.125	0.875	
1.44	0.250	0.75	
2.88	0.500	0.50	
5.76	1.00	0	
	0	0	1.0

Strict quality control and appropriate training of technical staff performing semen analysis and other laboratory procedures are important. All andrology technicians must be periodically trained to ensure uniformity, accuracy and reproducibility, especially in sperm count, motility and sperm morphology (Mortimer, 1994d). Internal quality controls, proficiency testing of slides for viability and morphology both by WHO and Kruger's strict criteria are available, and laboratories can enroll in a proficiency testing program. Technicians score the slides in a blind fashion, and the results are mailed to the proficiency testing program. Adequate remedial measures should be in place to minimize inter- and intraobserver variations. In addition, strict quality control for monitoring all equipment in use is critical, and all solutions and reagents must be subjected to strict quality control measures to optimize performance and accuracy of results.

CONCLUSION

Semen analysis is fairly simple and easy to perform and remains the cornerstone of the andrology laboratory even as newer tests are being introduced. Maintaining strict interlaboratory, inter-observer, and intra observer quality control measures and making quality control materials available is important for optimizing results.

BIBLIOGRAPHY

1. Anderson DJ. Should male infertility patients be tested for leukocytospermia? Fertil Steril. 1995;63:246-48.
2. Bronson R, Cooper G, Rosenfeld D. Sperm antibodies: their role in infertility. Fertil Steril 1984;42:171-83.
3. Cooper TG, Yeung CH, Nashan D, Jockenhövel F, Nieschlag E. Improvement in the assessment of human epididymal function by the use of inhibitors in the assay of alpha-glucosidase in seminal plasma. Int J Androl. 1990;13:297-305.
4. Davis JS, Gander JE. A reevaluation of the Roe procedure for the determination of fructose. Anal Biochem 1967;19:72-79.
5. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane

and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-28.
6. Johnsen O, Eliasson R. Evaluation of a commercially available kit for the colorimetric determination of zinc in human seminal plasma. Int J Androl 1987;10:435-40.
7. Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, Smith K. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril 1986;46:1118-23.
8. Moon KH, Bunge RG. Observations on the biochemistry of human semen. I. Fructose. Fertil Steril. 1968;19:186-91.
9. Mortimer, D. Antisperm Antibodies. In: Practical Laboratory Andrology. Oxford University Press, Oxford, 1994a;111-25.
10. Mortimer, D. Biochemistry of spermatozoa and seminal plasma. In: Practical Laboratory Andrology. Oxford University Press, Oxford, 1994c;89-109.
11. Mortimer, D. Semen microbiology and virology. In: Practical Laboratory Andrology. Oxford University Press, Oxford, 1994b;127-33.
12. Mortimer D. Technician training and Quality control Aspects. In: Practical Laboratory Andrology. Oxford University Press, Oxford, 1994d;337-47.
13. Sigman M, Jarow JP. Male infertility. In: Wein AJ eds. Campbell-Walsh Urology. 9th edition. Sanders Elsevier. Philadelphia, PA.2007;609-53.
14. Wolff H, Anderson DJ. Immunohistologic characterization and quantitation of leukocyte subpopulations in human semen. Fertil Steril 1988;49:497-504.
15. World Health Organization. WHO laboratory manual for the examination of human semen and sperm–cervical mucus interaction, 1999, 4th edn, Cambridge University Press, Cambridge.

CHAPTER 12

Sperm-Cervical Mucus Interaction Test and Its Importance in the Management of Infertility

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ABSTRACT

Sperm-cervical mucus penetration test is a preliminary diagnostic test useful for infertile couples with no obvious anatomical and physiological abnormality. Cervical mucus constitutes first selection barrier for the selection of the healthiest sperm for fertilization of the ovum.

The sperm-cervical mucus interaction test is useful in accessing the incapability of the hostile cervical mucus. This test can also help physicians in diagnosis of antisperm antibodies in the partner. This chapter presents the importance of this simple but useful test in the diagnosis and management of infertility.

INTRODUCTION

Infertility, defined as the inability to achieve pregnancy after one year of unprotected intercourse, often is associated with abnormal semen parameters in the man or menstrual abnormalities in the woman. In some cases, however, infertility is evident despite a normal semen profile in the man and a normal menstrual cycle in the woman with no apparent physiological defect. The explanation in such cases may lie in abnormalities of the cervix. The cervix has been shown to play an important role in the sperm's ability to permeate into the uterine cavity. Cervical abnormalities and hostility of the cervical mucus towards sperm are responsible for infertility in approximately 5-10% of women.[9] The sperm-cervical mucus penetration test has emerged as a useful method for an initial screening of couples with immunological infertility and husbands who developed antisperm antibody against the cervical mucus of their wives.

After intercourse, semen is deposited into the vagina where seminal coagulum liquefies and the active sperms are released, passing through a complex biological fluid known as cervical mucus that is produced from the secretory cells of the endocervix. The cervical mucus is considered to be the first selective barrier limiting the accessing active sperm to the female genital tract. As mucus covers the opening of the cervix, it prevents pathogens from ascending into the uterus. The fluid that is secreted into the vagina traps microorganism and flushes them out of the vagina, protecting both the uterine sterility and the vaginal epithelium.

Human cervical mucus consists of two parts; the aqueous part containing carbohydrates, amino acids, lipids, soluble macromolecules, locally produced proteins, peptides, polysaccharides, enzymes and hormones. The other component is a gel containing mucins, high molecular weight glycoprotein that forms a complex mesh-like structure. Throughout the menstrual cycle, the biophysical and biochemical characteristics of the mucus, as well as the quantity of the fluid, show significant changes. At midcycle, under the influence of estrogenic hormones, the amount of mucus increases, and it becomes more hydrated and less viscoelastic. These changes facilitate penetration by spermatozoa. When progesterone level increases, the mucus becomes less hydrated and more viscous and acts as a barrier to sperm. When mucus is most abundant, inorganic salts like NaCl along with K ions play an important role in forming organized crystallization, known as a ferning pattern.[1,2] Some studies also indicated that abnormally thick cervical

mucus that is resistant to sperm penetration is produced due to abnormal follicular growth, which is related to inadequate estrogen production.

Cervical mucus plays a major role in diagnosing sperm autoimmunity. Sperm cells with surface antibodies show impaired motility and agglutination, which hinders or prevents the penetration of spermatozoa through the cervical mucus. Several studies show a positive correlation between sperm autoimmunization and a poor result of the postcoital *in vivo* sperm-cervical mucus penetration test. On the other hand, three types of immunoglobulins (IgA, IgG, IgM) are present in cervical mucus that act as antibodies and may cause immunological reactions with sperm surface antigens. This might result in death or agglutination of sperm, leading to infertility. The cervical mucus penetration test enables the physician to identify the incompatibilities of hostile mucus and antisperm antibodies.

The use of human mucus for *in vitro* penetration tests presents several difficulties, including the problem of collecting relatively large quantities of the fluid from the endocervix and wide variation in viscosity.[2,3,5] Several substitutes for mucus are in use, including bovine cervical mucus, which has similar biophysical and biochemical properties and is available in large amounts and hyaluronic acid, which is a mucopolysaccharide that is structurally similar to human cervical mucus.[6] Other substitutes also have been proposed.

COLLECTION AND PRESERVATION OF CERVICAL MUCUS

Collection of cervical mucus from the endocervix is performed without anesthesia under aseptic conditions. All equipment including the speculum, forceps, cotton, etc. should be sterilized properly. The uterine cervix is exposed by sterilized speculum, and the exocervical region is thoroughly cleaned by sterile cotton swab to remove the exocervical mucus and vaginal contaminants. This should be done by a clinician or certified trained nurse. Mucus is obtained from the endocervix by gentle aspiration with the help of a plastic sterilized Pasteur pipette, sterile tuberculin syringe without needle or polyethylene tube, etc. During collection, suction should be maintained without causing trauma to the uterus. Bubble formation and vaginal interference should be avoided to ensure an accurate evaluation.

After collection, the properties of the mucus should be tested on the day of collection. If this is not practical, the sample must be preserved properly until it is tested. Mucus can be preserved directly in the aspiration tool or in a small microfuge tube that is sealed by plasticine to avoid dehydration, contamination with air or microorganisms if the storage container is stored at 4°C up to 5 days before analysis.

CERVICAL MUCUS EVALUATION

The fertile period can be predicted by the mucus quality across the ovulatory period. At an appropriate time during the menstrual cycle, sperm cells can migrate through the cervical mucus. This period of time varies among women and in the same individual from one cycle to another. Cervical mucus is an egg white-like viscous fluid, which increases in amount and normally takes a watery like appearance around the time of ovulation.

Cervical mucus evaluation includes assessment of volume, spinnbarkeit, fern ing [crystallization], consistency, cellularity and pH. A scoring procedure for cervical mucus was postulated by Moghissi in 1976,[7] based on the original proposal of Insler *et al.*[4] The pH of the cervical mucus is excluded from the scoring criterion. The final score is obtained by adding the individual scores of each category. A score greater than 10+ is considered good cervical mucus; a score less than 10+ is considered unfavorable cervical mucus. Maximum score is 15+.

Scoring Parameters

1. Volume

The volume is scored as follows:

0 = 0 ml
1+ = 0.1 ml
2+ = 0.2 ml
3+ = or > 0.3 ml

2. Consistency

Cervical mucus viscosity is measured by scoring consistency. Viscosity decreases during ovulation, which facilitates sperm penetrability. Higher viscosity cervical mucus that is more resistant to sperm migration is observed in the luteal phase of the menstrual cycle.

Consistency is scored as follows:

0 = thick, highly viscous, premenstrual mucus
1+ = mucus of intermediate viscosity
2+ = mildly viscous mucus
3+ = watery, minimally viscous, midcycle (preovulatory) mucus

3. Ferning

The crystallization pattern of cervical mucus is observed by spreading the mucus on a glass slide that is air-dried and observed under 400× magnification. This crystallization pattern, or ferning, is due to the presence of inorganic salts such as NaCl and K ions. Fern structure also varies with the composition of the cervical mucus. A primary stem and secondary, tertiary and quaternary branching are observed under the influence of hormones and ions of the cervical mucus.

Ferning is scored as follows:

0 = absent

1+ = initiation of fern formation

2+ = primary and secondary stems

3+ = tertiary and quaternary stems

(Figure 12.1)

4. Spinnbarkeit

Spinnbarkeit is evaluated by measuring the distance in centimeter that mucus can be stretched with the aid of forceps.

Spinnbarkeit is scored as follows:

0 = 1 cm

1+ = 1-4 cm

2+ = 5-8 cm

3+ = 9 cm or more

(Figure 12.2)

Figure 12.1: Human cervical mucus ferning with tertiary and quaternary stems

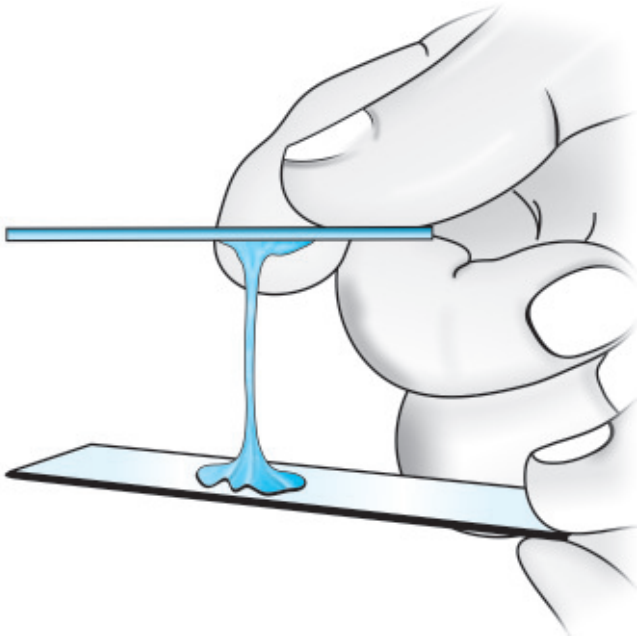

Figure 12.2: Spinnbarkeit test

5. Cellularity

Leucocytes and other cells present in the mucus are evaluated in terms of 0 to 3+

3+ = no cells are observed in each high power field

2+ = 1-10 cells/HPF

1+ = 11-20 cells/HPF

0 = more than 20 cells/HPF

The final score is obtained by adding the scores of the individual parameters.

pH

Cervical mucus pH is determined by placing a drop on pH paper. The normal pH range of midcycle cervical mucus is 7-8.5. Proper assessment of cervical mucus pH is necessary, as it significantly affects sperm motility and viability. Slightly alkaline mucus facilitates sperm motility; acidic pH may indicate abnormal secretion of cervical mucus or bacterial infection. The possibility of contamination through vaginal secretion due to improper and careless collection methods should always be ruled out.

Post-coital Test (PCT)

This is an *in vivo* cervical mucus interaction test. The most appropriate time for the test is 2-5 hrs after intercourse, since the largest sperm population in the mucus is

normally found at this time. The post-coital test should be performed prior to ovulation, with the optimal timing just before ovulation. Ovulation can be predicted by clinical investigation, such as basal body temperature, cervical mucus changes, and ultrasound results for follicular size determination or hormonal status. The purpose of the study is to evaluate sperm survivability and activity, as well as cervical mucus hostility.

Procedure for PCT

Patients are instructed to abstain from intercourse for at least 2 days before the day of the test, and to avoid the use of lubricants during or after intercourse or soap for bathing. A nonlubricated, sterilized speculum is inserted to expose the vagina. The vaginal sample should be cleared thoroughly by a tuberculin syringe/plastic Pasteur pipette or a polyethylene tube, which should be examined to ensure semen deposition. After the mucus sample is aspirated from the endocervical canal, it is placed onto a clean slide and covered with a cover slip, avoiding bubble formation. The sample is examined under light microscope at 100 × followed by examination at 400 ×. The number of spermatozoa in the mucus is counted by taking 20 fields, and a mean is taken.

Evaluation of Sperm Concentration and Motility

PCT is considered "good" when at least two motile spermatozoa of high progressive motility are observed in preovulatory cervical mucus, 8-12 hrs after intercourse; otherwise, it is classified as poor.[2] For further detailed evaluation and reporting, PCT results are subdivided in four groups:

Negative: No spermatozoa found in mucus/LPF, but present in vaginal secretion.

Inadequate: Two motile sperm/HPF

Moderate: Two to six motile sperm/HPF

Excellent: Seven or more motile sperm/HPF.

Motility of spermatozoa is graded as follows:

a = rapid progressive motility
b = slow or sluggish progressive motility
c = nonprogressive motility
d = immotile spermatozoa

The presence of any rapidly progressive motile spermatozoa in the cervical mucus is an indicator of normal spermatozoa. Negative or abnormal PCT results may be observed due to improper deposition of semen into the vagina or incorrect timing. In the event of a negative or abnormal result, the PCT should be repeated several times during the same cycle.

In Vitro Cervical Mucus Penetration Tests

Negative or abnormal PCT results are an indication for performing *in vitro* cervical mucus penetration tests. Two different techniques have been used for *in vitro* investigation of sperm penetration: (1) The simplified slide method (2) the capillary tube technique. The *in vitro* sperm mucus penetration test (SMPT) is done to measure the ability of the spermatozoa to migrate into a column of mucus or substitute.[8]

In vitro Test

The *in vitro* test is performed after repeated abnormal post-coital test results. This test is more useful when donor semen and donor cervical mucus are used to verify the source of the abnormal/incorrect results obtained using both the husband's sperm and the wife's cervical mucus.

Sample Collection

A semen sample is collected from the husband or a male donor after abstinence from intercourse at least for 2 days. Cervical mucus is collected from the wife or a female donor who is undergoing artificial insemination in her natural cycle or is being treated by gonadotrophin. Patients taking clomiphine citrate are excluded from the study because of its possible effect as an antiestrogen on the cervix. The test ideally should be done within 30 to 60 minutes after semen collection, Collection of a midcycle human cervical mucus under sterile condition is essential for proper assessment.

Simplified Slide Test

A moist chamber is prepared for placement of the glass slide. A drop of cervical mucus is placed on a clean glass slide and covered with a cover slip (22 mm × 22 mm), avoiding bubble formation. A drop of semen is deposited at the each side and in contact with the cover slip so that semen can move into the mucus by capillary action. The slide is placed in the moist chamber and kept at 37°C for 30 min. After 30 minutes, it is observed under bright field microscope where a finger-like projection is observed at

the semen-mucus interface. Spermatozoa enter into the cervical mucus after crossing the interface. This is a qualitative method, and this test cannot be used to quantify the number of cells present in the mucus or to measure the size and shape of the interface.

It is scored as follows:

Normal: > 90% cells are motile with rapid forward movement.

Poor: Slow progressive motile cells.

Abnormal: Most of the sperm cells are immotile, lack forward progression or exhibit shaking movement, which may indicate the presence of antisperm antibodies.

Zero: No penetration is observed.

Capillary Tube Test

This test measures the ability of sperm in the semen to swim up into a column of cervical mucus.

Method

A flat capillary tube 5 cm × 3 mm × 0.3 mm is normally used for this test. Midcycle human cervical mucus is aspirated directly into the capillary tube, avoiding bubble formation. One end of the tube is sealed with plasticine, and the open end of the tube is placed in a 1.5 ml microfuge tube (Appleton Woods, Birmingham, UK) containing 100 µl of the liquefied semen sample. The semen reservoir and the capillary tube are placed on a microscope slide in horizontal position as described in the WHO manual (1999). After incubation for 1 hour at room temperature while maintaining the humidity, the capillary tube is observed under bright field illumination with 200 × magnification, Scanning of the capillary tube under the microscope establishes the distance furthest from the semen reservoir attained by the spermatozoa. The migration distance is defined as the maximum distance in centimeters covered by spermatozoa after 1 hour of incubation. Sperm penetration concentration is assessed at half the migration distance using the same magnification and counting spermatozoa while focusing from the lower to upper wall of the capillary in a single pass. The penetration score is calculated by multiplying half the migration distance by the number of spermatozoa counted at half the migration distance. Sperm motility is assessed by examining at least 200 spermatozoa at half the migration distance.[10,11]

REFERENCES

1. Bothner H, Wik O. Rheology of hyaluronate. Acta Otolaryngol 1987;442 (suppl.):25-30.
2. Eggert-Kruse W, Gerhard I, Tilgen W, Runnebaum. B. Clinical significance of crossed *in vitro* sperm-cervical mucus penetration tests in infertility investigation. Fertil Steril 1989a;52:1032-40.
3. Eggert- Kruse W, Leinhos G, Gerhard I, Tilgen W, Runnebaum B. Prognostic value of *in vitro* sperm penetration into hormonally standardized human cervical mucus. Fertil Steril 1989b;51:317-23.
4. Insler V, Melmed H, Eichenbrenner I, Serr DM, Lunenfeld B. The cervical score. A simple semiquantitative method for monitoring of the menstrual cycle. Int J Gynaecol Obstet 1972;10:223-8.
5. Karni Z. Newtonian viscosity of the human cervical mucus during the menstrual cycle. Int J Fertil 1971;16:185-8.
6. McDonald RR. Cyclic changes in cervical mucus. J Obsterr Gynecol Brit Com 1969;76:1090.
7. Moghissi, KS. Post-coital test: physiological basis, technique and interpretation. Fertil Steril 1976;27:117-29.
8. Ola B, Afnan M, Papaioannou S, Sharif K, Lars Bjorndahl L Coomarasamy A. Accuracy of sperm-cervical mucus penetration tests in evaluating sperm motility in semen; a systematic quantitative review. Hum. Reprod 2003;18:1037-46.
9. Sims JA, Gibbons WE. Treatment of human infertility, the cervical and uterine factors. In: Adashi EY, Rock JA, Rosenwaks Z [editors] Reproductive endocrinology, surgery and technology. Lippincott-Raven, New York. 1996;2142-69.
10. Tang S, Garrett C, Gordon-Baker HW: Comparison of human cervical mucus and artificial sperm penetration media. Hum Reprod 1999;14:2812-17.
11. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. World Health Organization 1999; 4th edn: Cambridge University Press, Cambridge.

CHAPTER 13

The Clinical Importance of Sperm Morphology in Assisted Reproduction

Daniel R Franken

INTRODUCTION

The first description and illustration of the human sperm cell was done by van Leeuwenhoek in 1677. Until the 20th century, very little attention was given to sperm morphology assessment, and consequently as a result of the lack of uniformity in human sperm morphology evaluation, multiple rating modules were published. The first set of standardized criteria for sperm morphology was established by MacLeod and Gold (1951), while others made important contributions towards standardization.[1-3]

The realization and concept of a normal sperm and its role during fertilization initiated in the middle 70s during a survey on 1004 couples attending the Infertility Clinic at Tygerberg Hospital. The founders of the sperm morphology concept at Tygerberg Infertility Clinic, Drs JA van Zyl and R Menkveld divided the data of the survey using the percentage normal forms present in the ejaculates as discriminator. The study showed a linear increase in natural fertilization corresponding with a rise in the percentage normal sperm morphology.[4] It was later statistically defined that the critical threshold of normal morphology for success for fertilization *in vitro* as well as *in vivo* was 4%.[5,6] During these studies the appearance of normal forms were described based on sperm cells selected by postcoital cervical mucus or from the surface of the human zona pellucida.[7] These statements were confirmed by Coetzee et al,[8] in 1998 who summarized all the important articles at that time period in a meta-analysis. That study concluded that the inclusion of an accurate evaluated normal sperm morphology count as an integral part of the standard semen analysis, makes the analysis still the most cost-effective means of evaluating the male factor.[8]

An important breakthrough was noted in 1999 with the publication of the World Health Organization's guidelines for semen analysis, which embraced the Tygerberg strict criteria classification scheme as the method of evaluating human sperm morphology.[7] In that publication a spermatozoon is considered normal when the head, neck/midpiece and the tail was normal.

What is a Normal Sperm Cell?

The definition of a normal sperm cell includes specific guidelines such as; first and foremost; the head should be oval with a smooth configuration. The acrosome should be well defined comprising about 40-70% of the sperm head. Allowing for the slight shrinkage that fixation and staining induce; the length of the head should be 4.0-5.0 µm (median 4.1 µm) and the width 2.5-3.5 µm (median 2.8 µm). The length-to-width ratio should be 1.50 to 1.75. Although this criteria is often disregarded, our experience convinced us that once the reader is skilled in morphology evaluation, the ratio can become extremely useful during the selection process during ICSI therapy.[9] The midpiece should be slender, less than 1 µm in width, about one and a half times the length of the head, and attached axially to the head.[7] The tail should be straight, uniform, and thinner than the midpiece, uncoiled and approximately 45 µm long (Figure 13.1). Once a normally shaped spermatozoon is identified, an eyepiece micrometer may be useful for distinguishing between the

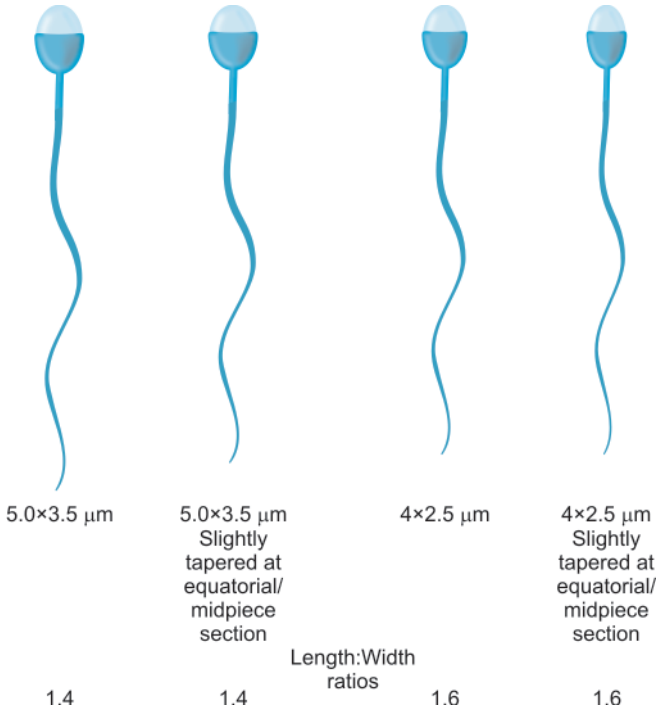

Figure 13.1: Four basic normal forms of human sperm as classified by strict Tygerberg criteria with morphometrical information and length:width ratios

normal and abnormal spermatozoa, but with this technique the form of the sperm head is more important than its dimensions unless grossly abnormal. Although some may regard the use of an ocular micrometer as unnecessary, the estimation of the length and width of the spermatozoa are extremely valuable for the untrained observer.

Sperm aberrations can be classified into 3 categories namely; defects of the head, neck/midpiece and tail. Head defects include large or small, tapered, pyriform, round, amorphous, vacuolated (>2 vacuoles or >20% of the head area occupied by unstained vacuolar areas), vacuoles in the postacrosomal region, heads with small or large acrosomal areas (<40% or >70% of the head area), double heads, or any combination of the above aberrations (Figure 13.2).

Neck and midpiece defects comprise of sperm with an asymmetrical insertion of the midpiece into the head, thick or irregular, sharply bent, abnormally thin, or any combination of these tail defects include short, multiple, hairpin, broken, sharply bent, of irregular width, coiled, or any combination of these excess residual cytoplasm: this is associated with abnormal spermatozoa produced from a defective spermatogenic process (Figure 13.3).

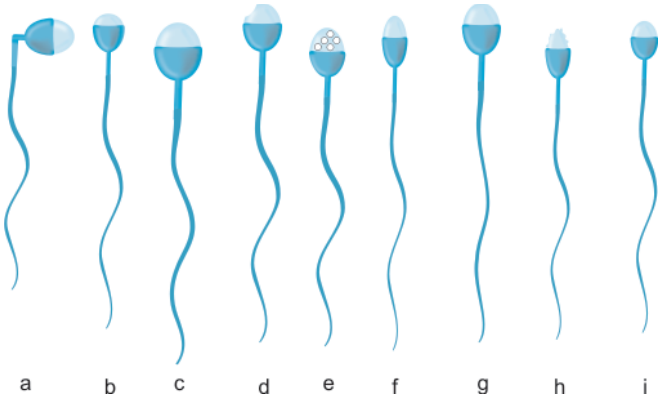

Figure 13.2: Diagrams of some typical sperm head and midpiece aberrations a: bent neck; b: small acrosome; c: macrocephal; d: irregular surface; e: vacuoles; f: tapered; g: thin midpiece; h: amorph; i: microcephal

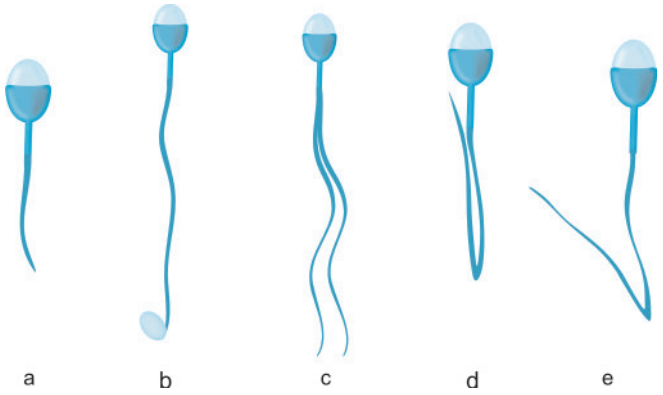

Figure 13.3: Diagrams of some typical sperm tail aberrations a: stumped tail; b: bent tail; c: droplet; d: duplicated tail; e: hairpin tail

Spermatozoa characterized by large amounts of irregular, stained cytoplasm, often associated with defective midpieces, and one-third or more of the sperm head size[10] are abnormal. This excess cytoplasm should no longer be called a cytoplasmic droplet.[11]

The Importance of Training

Since more clinicians are becoming aware of the importance of training and subsequent quality control measurements, standardization in semen analysis methodologies has become mandatory. In close agreement with the present author's beliefs have made an important contribution towards the standardization of techniques needed to obtain a globally accepted and World Health Organization recognized semen analysis result.[12]

Knowledge and understanding of the morphological appearance and bright-field microscopic configuration

of a normal human sperm cell form the basis of the strict criteria evaluation method. Our experience has clearly indicated that discrepancies in the reports are mainly due to the lack of knowledge and the urgent need for training.[13-17] We and others have shown that with proper training, technologists can acquire sperm morphology reading skills that are reliable and consistent over an extended period.[18,19] The findings of these studies can be regarded as a wake-up call for centers that provide semen analyses for referring clinicians. If we regard these findings as representative of medical laboratory scientists' sperm morphology reading skills, clinicians in developing countries should be concerned about the diagnostic quality as far as male infertility is concerned. It is well recognized that the evaluation of the percentage of normal sperm morphology features with light microscopy is subjective and therefore, difficult to compare between laboratories and even within laboratories.

Clinical Relevance

The development of *in vitro* fertilization (IVF) procedures as a novel infertility therapy directly caused the establishment of a model where certain sperm variables such as morphology and IVF could be studied.[5] The model for sperm morphology became known as Tygerberg's strict criteria or the Kruger criteria. The model is mainly based on the appearance of a normal sperm cell. In that study, all male partners had >20 × 10^6 sperm/mL and motility >30% to eliminate all the possible impact of other semen parameters. The results of that study were responsible for the initiation of multiple publications and discussions on the impact of sperm morphology on human *in vitro*[20] and *in vivo*[6] fertilization.

In Vitro Fertilization

During large prospective studies reporting on the data obtained from 199 cycles, logistic regression analysis was used to determine the thresholds for fertilization using sperm morphology as a contributing factor.[5,20] It was reported in cases with <5% (previously defined as 0-4%) normal forms, the fertilization rates were 7.6%. The fertilization rate in the 5-14% group was 63% and the >14% group 88%.[8,20,21] The impact of sperm morphology on IVF was reported by Coetzee et al, in 1998 during a structure literature review. The statistical outcomes and final conclusions of the review were analyzed and tabulated and where sufficient data were available, the odds ratio for fertilization per oocyte and pregnancy per cycle were calculated.

Using the suggested 5% threshold as a cut of value, 49/216 studies, the majority, i.e. 82% of the analyzed studies concluded that normal sperm morphology had a role to play in the diagnosis of male fertility potential. With a 5% threshold, ten studies provided data that could be analyzed for prediction of fertilization, while 11 studies could be used to predict pregnancy. Except for one study all had a positive predictive value for *in vitro* fertilization. All the studies showed a positive predictive value for fertilization *in vitro* except one (odds ratio 1.42 CI; 0.9 to 2.25) not reaching significance.[21]

Intrauterine Insemination

Intrauterine insemination as an infertility therapy has regained its use due to the improvement of the sperm preparation and washing procedures. The washing procedures are necessary to remove prostaglandins, infectious agents and antigenic proteins present in the seminal fluid. Another substantial advantage of these techniques is the removal of nonmotile sperm leucocytes or immature germ cells. Despite the extensive literature on the subject of artificial homologous insemination, controversy still surrounds the effectiveness of this very popular treatment procedure. This dilemma might be caused by the studies reporting on the poor results obtained when IUI was performed in natural cycles. This is true not only for male factor subfertility indications, but also for unexplained and cervical factor subfertility.[22]

The treatment became more popular and effective when controlled ovarian hyperstimulation (COH) was introduced.[23] The rationale behind the use of COH is the increase of the number of oocytes available for fertilization and to correct subtle unpredictable ovulatory dysfunction.[22] IUI is cheaper, simpler and less invasive than the more sophisticated assisted reproductive methods of IVF and ICSI. Intrauterine insemination is therefore, often offered to couples as a first line of approach in cases of hostile cervical mucus, antisperm antibodies or idiopathic infertility. van Waart et al, 2001 conducted a structured review recording the predictive of normal sperm morphology in intrauterine inseminations.[24] (van Waart et al, 2001).

In that study eighteen articles that mentioned a clear predictive value of normal sperm morphology were

divided into a Tygerberg strict criteria group[5,7] and a WHO[25,26] group. These groups were chosen since the literature often reflected on results for sperm morphology using either one of these classification systems. The main difference between these two systems are the reported cut-off values for normal sperm morphology, namely <14% and <4% for Tygerberg strict criteria and 30% for WHO 1992/1999 method. Six of the nine articles that used Tygerberg's strict criteria; reported a positive value for sperm morphology, while three stated no predictive value. The articles that used the WHO criteria noted similar values for morphology.[24,26] However, eight of these reports have sufficient data to reanalyze statistically; six used Tygerberg criteria and two the WHO criteria. A meta-analysis of the six studies that used Tygerberg criteria yielded a risk difference between the pregnancy rates achieved in the couples below and above 5% strict criteria threshold of –0.07 (95% CI: –0.1 to –0.03; $p < 0.001$). The meta-analysis thus indicated a significant improved pregnancy rate above or equal to the 5% strict criteria threshold.

Similar results were reported by Ombelet and colleagues in a previous study.[22] In a review of 1100 intrauterine insemination treatment cycles, the authors found a significant difference in cycle fecundity when comparing cases with <5% normal forms with the group >5% normal forms. This relationship between sperm morphology and IUI was also demonstrated by Eggert-Kruse and colleagues.[6] In conclusion, most of the published work are in support and emphasizes the importance of sperm morphology as a predictor for men's fertilizing potential *in vivo*.

Intracytoplasmic Sperm Injection (ICSI)

Sperm morphology has been described and identified as an excellent biomarker of human sperm dysfunction to assist clinicians to identify the source of infertility among male factor men.[27-29] After the onset of ICSI however, the fertilization rates obtained with semen samples of poor morphology (<5% normal cells) did not differ from those obtained with semen samples with better overall sperm morphology.[30-32] Morphology therefore, did not seem to play a role after injected into the cytoplasma of the mature oocyte.

On the other hand, the use of teratozoospermic semen was also correlated with poor or very low implantation and ongoing pregnancy rates. However, reports were published describing simple but effective methods to select sperm for ICSI therapy.[33] In short, these studies by de Vos *et al*, reported no difference in clinical pregnancy rates. The authors explained this observation by suggesting that the embryologist selects a motile, normal-looking spermatozoon for injection into the oocyte. As long as morphologically well-shaped live sperm cells can be used for injection, fertilization and pregnancy rates after ICSI are not affected.

Similar results were reported by Raja and Franken[9] who concluded that the sperm selection process for ICSI based on the approach of choosing the 'best-looking' spermatozoon in the ejaculate seems to provide cells that can be classified as normal based on the length-to-width ratio set by the World Health Organization for normal cells. Although the sperm selection is done at low magnification (400 ×) the basic shape as defined by strict criteria of the sperm, i.e. smooth oval shape with normal length:width ratio (1.5 : 1.7) is clearly visible for the trained observer.

The never-ending search for a method that will provide the possibility to select the best sperm that would be able to provide viable embryos, lead to an increase in the investigations of the quality of gametes. Recently, a new method of intracytoplasmic morphologically selected sperm injection has been proposed, based on motile sperm organellar examination that results in a higher pregnancy rate and a decreased incidence of abortion. Bartoov and colleagues[34] devised a novel method for sperm selection by evaluation of sperm during ICSI with use of an inverted light microscope equipped with high-power Normansky optics enhanced by digital imaging to achieve a magnification up to 6300 × and permitting insemination using spermatozoa carrying morphologically normal nuclei.

A new and very exciting technique involves sperm birefringence assessed by an inverted microscope equipped with Hoffman contrast and polarizing and analyzing lenses.[35] The technique is based on the birefringement characteristics.[36] of the sperm cells caused by anisotropic properties of their protoplasmic composition. The morphologic parameters of the sperm samples included in the study were analyzed according to the criteria established by the World Health Organization[25] and, for the morphology by Kruger et al[5] (Table 13.1).

Table 13.1: Birefringement characteristics of men undergoing ICSI therapy

Group	*Percentage of birefringement*
Normozoospermic	87.0 ± 11%
Oligoasthenozoospermic	55.3 ± 27.6%
Teratozoospermic	22.1 ± 17.0%
TESE	13.8 ± 9.1%

CONCLUSION

During the last six decades the criteria for the evaluation of sperm morphology has changed substantially. It was only during the last 10 years that an international awareness was noted indicating that some form of standardization has been reached. This is especially true when we consider the WHO's recognition and acceptance of strict criteria as the method of morphology evaluation as a mean to standardize. Nevertheless, whether sperm cells are evaluated by means of bright field microscopy, computer assisted sperm analyzing systems, Normansky optics or Hoffman contrast and polarizing and analyzing lenses, the criteria to be investigated remains morphology in all cases.

REFERENCES

1. Freund M. Standards for rating human sperm morphology. Int J Fertil. 1966;11:97-118.
2. Eliasson R. Standards for investigation of human semen. Andrologie. 1971;3:49-64.
3. David G, Bisson JP, Czyglik F, et al. Anomalies morphologiques du spermatozoide humain. 10 Propositions pour un systeme de classification. J Gynecol Obstet Biol Reprod. 1975; 4 (Suppl 1): 17-36.
4. Van Zyl JA, Kotze TJ, Menkveld R. Predictive value of spermatozoa morphology in natural fertilization. In Acosta et al. eds. Human Spermatozoa in Assisted Reproduction, Baltimore Williams & Wilkins. 1990;319-24.
5. Kruger TF, Menkveld R, Stander FSH, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril. 1986;46:1118-23.
6. Eggert-Kruse W, Reimann-Anderson J, Rohr G, et al. Clinical relevance of sperm morphology assessment using strict criteria and relationship with sperm-mucus interaction in vivo and in vitro. Fertil Steril. 1995;63:612-24.
7. World Health Organization. WHO laboratory manual for examination of human semen and semen-cervical mucus interaction. 4th ed. Cambridge University Press. Cambridge, United Kingdom 1999;14-7.
8. Coetzee K, Kruger TF, Lombard CJ. Predictive value of normal sperm morphology: a structured literature review. Hum Reprod Update. 1998; 4:73.
9. Raja K, Franken DR. The influence of Y chromosome bearing sperm and sperm morphology on the outcome of intracytoplasmic sperm injection. Andrologia 2006;38:179-83.
10. Mortimer D, Menkveld R. Sperm morphology assessment – historical perspectives and 9354 current opinions. J Androl 2001;22: 192-205.
11. Cooper TG. Cytoplasmic droplets: the good, the bad or just confusing? Hum Reprod. 2005;20:9-11.
12. Kvist U, Bjorndahl L, (Eds). Manual on Basic Semen Analysis. Oxford University Press, Oxford 2002.
13. Franken DR, Menkveld R, Kruger TF, et al. Monitoring technologist reading skills in a sperm morphology quality control program. Fertil Steril. 2003;1637-43.
14. Franken DR, Dada OA. The establishment of sperm morphology satellite training laboratories in Africa. Andrologia 2004;37:57-60.
15. Franken DR, Avari K, Palshekar N. Sperm morphology training is mandatory to ensure relevant clinical results. Andrologia 2008;40: 377-80.
16. Franken DR, Kruger TF. Lessons learned from a sperm morphology quality control programme. Andrologia 2006;38:1-5.
17. Franken DR, Aneck-Hahn N. Sperm morphology training programmes: the African experience, Hum Reprod Monograph 2008:1.
18. Cooper TG, Atkinson AD, Nieschlag E. Experience with external quality control in spermatology. Hum Reprod. 1999;14:765-9.
19. Franken DR, Kruger TF. What is a normal spermatozoon? In Atlas of Human Sperm Morphology Evaluation (Eds) Kruger TF, Franken DR. Parthenon Press, London, 2004.
20. Kruger TF, Acosta AA, Simmons FF, et al. Predictive value of abnormal sperm morphology in in vitro fertilization. Fertil Steril. 1988; 49:111-7.
21. Kruger TF, Van der Merwe F, van Waart J. The Tygerberg strict criteria: what are the clinical thresholds for in vitro fertilization, intrauterine insemination and in vivo fertilization? In: Atlas of Human Sperm Morphology Evaluation. Kruger TF, Franken DR (Eds). Taylor and Francis, London, 2004:13-8.
22. Ombelet W, Vanderput H, Van de Putte G, et al. Intrauterine insemination after ovarian stimulation with chlomiphene citrate: predictive potential of inseminating motile count and sperm morphology. Hum Reprod 1997;12:1458-62.
23. Irianni FM, Ramey J, Vaintraub MT, et al. Therapeutic insemination improves with gonadotropin stimulation. Arch Androl 1993;31:55-72.
24. Van Waart J, Kruger TF, Lombard CJ, et al. Predictive value of normal sperm morphology in intrauterine insemination (IUI): a structured literature review. Hum Reprod Update 2001;7:495-500.
25. World Health Organization. WHO laboratory manual for examination of human semen and semen-cervical mucus interaction. 2nd (edn)., Cambridge University Press: Cambridge, United Kingdom, 1992.

26. Duran HE, Morshedi M, Kruger T, et al. Intrauterine insemination: a systematic review on determinants of success. Hum Reprod Update. 2002;8:373-84.
27. Acosta AA, Oehninger S, Morshedi M, et al. Assisted reproduction and treatment of male factor. Obstet Gynecol Surg. 1989;44:1-18.
28. Franken DR, Kruger TF, Menkveld R, et al. Hemizona assay and association of teratozoospermia, chromosomal aberrations and teratozoospermia: increasing sperm insemination concentrations to enhance zona pellucida binding. Fertil Steril 1990;54:497-503.
29. Oehninger SC, Kruger TF. The diagnosis of male infertility by semen quality. Hum Reprod. 1995;10:1037-41.
30. Tasdemir I, Murat T, Tavukcuog S, et al. Effect of abnormal sperm head morphology on the outcome of intracytoplasmic sperm injection in humans. Hum Reprod. 1997;12:1214-17.
31. Cohen J, Alikani M, Munne S, et al. Micromanipulation in clinical management of fertility disorders. Semi. Reprod Endocrinol. 1994; 12:151-6.
32. Mansour RT, Aboulghar MA, Serour GI, et al. The effect of sperm parameters on the outcome of intracytoplasmic sperm injection. Fertil Steril. 1995;64:982-6.
33. 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;79:42-8.
34. Bartoov B, Berkovitz A, Eltes F, Kogosovski A, Yagoda A, Lederman H, Artzi S, Gross M, Barak Y. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 2003;80:1413-9.
35. Baccetti B. Microscopical advances in assisted reproduction. J Submicrosc Cytol Pathol 2004;36:333-9.
36. Gianaroli L, Magli MC, Collodel G, et al. Sperm head's birefringence: a new criterion for sperm selection. Fertil Steril 2008;90:104-12.

CHAPTER 14

The Role of Leukocytospermia in Male Infertility

Ashok Agarwal, James Savia, Andrew Luu

ABSTRACT

Many studies have implicated the role of reactive oxygen species in male infertility. While reactive oxygen species has a physiological role in maintenance and function of the spermatozoa, excessive levels above the sperm defense leads to detrimental effects on sperm function and integrity. These possible detrimental effects are termed oxidative stress.

Ever since leukocytes were discovered in semen, it has been suggested as a source for the reactive oxygen species found in semen samples. Evidence indicates that leukocytospermia, which is an abnormally high amount of leukocytes in the semen (>1.0×10^6 leukocytes/ml of semen), is associated with poor semen parameters. Leukocytospermia can be symptomatic when it is a result of a urinary tract infection, such as prostatitis, but it can also be asymptomatic. With multiple etiologies, from bacterial causes to idiopathic causes, the methods of treatment for leukocytospermia include antioxidant and antibiotic supplementation. The success of these treatments, however, is greatly debated. Nonetheless, understanding the role of leukocytospermia in male factor infertility is essential because of the increasing evidence that oxidative stress does produce detrimental effects on sperm quality.

INTRODUCTION

Infertility is a major concern for approximately 15% of reproductive age couples (Agarwal, Nallella, et al 2004). The inability to conceive naturally can stem from many factors, including both male and female factor infertility. Approximately 10-20% of the cases of male factor infertility are being attributed to leukocytospermia (Sharma, Pasqualotto et al 2001); the remaining cases are being attributed to a variety of other factors, including varicocele, genetic, and systemic diseases.

Leukocytospermia is a condition in which there is an increase in the number of leukocytes in the ejaculate (Aziz, Saleh et al 2004). Leukocytes are white blood cells, which play a normal physiological role in the human immune system. Their focus is in response to both infection and foreign materials (Aziz, Saleh et al 2004). There are two main types of leukocytes: granulocytes, which include neutrophils, basophils and eosinophils, and agranulocytes, which include lymphocytes, monocytes and macrophages. These cells function as a defense system by utilizing an oxygen-based system to kill bacteria and other pathogens (Agarwal, Saleh et al 2003).

Leukocytes are normally present in the male reproductive tract and are seen in every human ejaculate. Ongoing research is currently investigating the clinical significance of increased leukocytes in semen and its relationship to male factor infertility. The World Health Organization (WHO) defines leukocytospermia as that greater than or equal to six million leukocytes present in the semen. Numerous studies support the notion that an increase of leukocytes in the semen is detrimental to sperm parameters such as morphology and motility (Erenpreiss, Hlevicka et al 2002; Agarwal, Saleh et al 2003; Moskovtsev, Willis et al 2007). In recent years, leukocytospermia has been shown to have adverse effects on sperm parameters through various mechanisms. A study conducted by Lackner et al (2008) demonstrated that 40% of infertile men with untreated leukocytospermia had an increase in negative semen parameters over a three month period. It has been postulated that an increase in leukocytes in the ejaculate

leads to an increase in reactive oxygen species (ROS) and subsequent oxidative stress (OS) (Lackner, Lakovic et al 2008). Both ROS and OS have long been implicated in sperm dysfunction by way of lipid peroxidation (Sharma and Agarwal 1996). Lipid peroxidation negatively impacts the polyunsaturated fatty acids in the sperm cell membrane thereby altering fluidity of the membrane and inflicting DNA damage. Unfortunately, mature sperm lack repair mechanisms after undergoing the maturation process. These cumulative effects lead to decreased sperm motility, abnormal sperm morphology and an overall decrease in sperm fertilization capacity and viability.

Our goal is to shed insight on the topic of leukocytospermia, and elucidate the relationship between leukocytospermia and infertility. Furthermore, we aim to provide a reference for andrologists on the laboratory and clinical aspects related to leukocytospermia.

Physiological Function of Reactive Oxygen Species

ROS are highly reactive oxygen molecules with one or more unpaired electrons. ROS are produced as normal byproducts of oxygen metabolism and function in everyday human cell-to-cell interactions, as seen in leukocytes (Agarwal, Makker et al 2008). Major ROS include hydrogen peroxide, superoxide anion, hydroxide, and nitric oxide. These ROS have both positive and negative impacts on sperm function depending on the level of concentration.

Under normal physiological conditions, ROS facilitate maturation and subsequent fertilization of the sperm. As sperm are ejaculated into the female reproductive tract, they must undergo a series of physiological processes such as capacitation, hyperactivation and acrosomal reaction in order for fertilization to occur. Small, basal quantities of ROS are necessary for these physiological processes to take place.

Aitken and his colleagues (1989) observed the limited amount of lipid peroxidation generated by basal levels of ROS increased sperm binding to the zona pellucida, thereby enhancing the acrosome reaction. In addition, Aitken et al (1989) demonstrated that by adding alpha-tocopherol, an antioxidant which acts to neutralize ROS, sperm binding was greatly reduced. Binding of sperm to the zona pellucida is essential for the acrosome reaction to occur. An impaired acrosome reaction as a result of lower than optimal ROS concentration negatively impacts the fertilization process. The results of this study are consistent with suggesting a possible physiological role for ROS in mediating sperm-zona pellucida interaction (Aitken, Clarkson et al 1989).

De Lamirande et al (de Lamirande, Leclerc et al 1997) further defined the physiological function of ROS in sperm physiology. They observed that ROS in low and controlled concentrations played a critical role in the processes of capacitation, hyperactivation, and the acrosome reaction, *in vitro.* Sperm hyperactivation and capacitation are a set of complex signal transduction reactions that occur in the female reproductive tract and allow sperm to mature and achieve fertilization capacity (de Lamirande, Jiang et al 1997). The experimental conclusions gathered by de Lamirande et al (1997) were demonstrated after observing spermatozoa exposed to both superoxide anion and superoxide dismutase (SOD). In the presence of superoxide anion, higher hyperactivation and capacitation were observed. Spermatozoa exposed to SOD showed the opposite effect-hyperactivation and capacitation were prevented (de Lamirande, Jiang et al 1997).

As demonstrated by both Aitken et al and De Lamirande et al, it is suggested that ROS positively impacts sperm physiology at controlled concentrations. Both research teams investigated this impact by observing spermatozoa in the presence of ROS and antioxidants. Recent research, however, is exploring the association between higher ROS concentrations, sperm pathology, and the resultant deleterious effects on male reproduction.

Pathological Role of Reactive Oxygen Species and Leukocytes

The male reproductive tract contains a variety of cells present in various stages of maturation. Of the numerous cell types, leukocytes and immature or abnormal spermatozoa have been implicated as the two main sources of ROS production (Sharma and Agarwal 1996). Leukocytes have a normal physiological presence in the male reproductive tract. They play a role in immuno-surveillance and inflammatory processes. Unfortunately, leukocytes in excess have been linked to poor semen quality and function (Wolff 1995). Peroxidase-positive leukocytes such as polymorpho-nucleocytes (PMNs), macrophages, and neutrophils have the ability to generate ROS via respiratory burst. Peroxidase-positive leukocytes are activated by various stimuli, such as inflammation, infection, and defective or dead sperm (Pasqualotto, Sharma et al 2000). Activation of the respiratory burst is

preceded by an increase in NADPH and myeloperoxidase systems which create high levels of ROS. This process by peroxidase-positive leukocytes is an early defense mechanism to fight infection. At physiological conditions, ROS production is counter balanced by antioxidants. When either an increase in ROS or a decrease in antioxidants occurs, an imbalance ensues and creates a condition of oxidative stress (OS) (Agarwal and Prabakaran 2005). OS can arise from a variety of pathologies that can be subdivided into two groups: as a direct result of primary pathologies to the male reproductive system or as an indirect result of systemic pathologies (Figure 14.1). OS leads to many deleterious effects on sperm parameters. One of the main mechanisms of action that OS leads to sperm damage is lipid peroxidation (Figure 14.2). Lipid peroxidation is an oxidative weakening of membrane polyunsaturated fatty acids. Human spermatozoa are highly susceptible to lipid peroxidation because of their high concentrations of polyunsaturated fatty acids in their plasma membrane (Griveau and Le Lannou 1997). The ROS attacks the polyunsaturated fatty acid carbon-carbon double bonds and leads to membrane disorganization and loss of fluidity (Agarwal and Saleh 2002). As a result, the ability of the sperm to attach to an oocyte is severely decreased.

Evidence also suggests that spermatozoa can produce ROS (Aitken, Buckingham et al 1992). Gomez et al (1998) correlated a decrease in sperm quality led to an increase

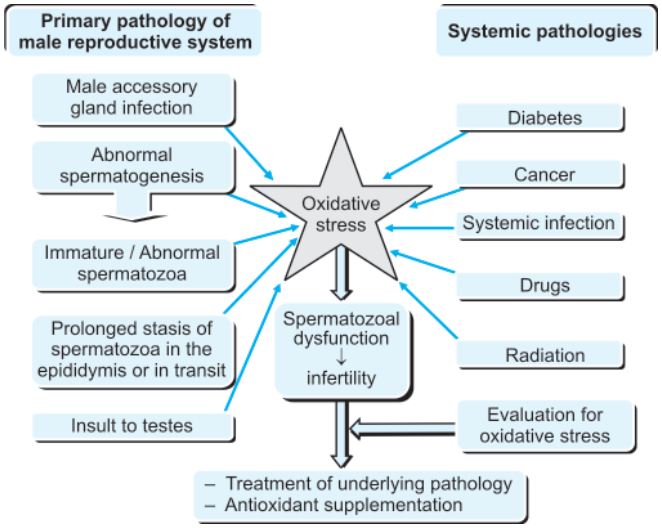

Figure 14.1: Summary of the pathologies for oxidative stress. After evaluation of the possible causes for the increase oxidative stress, a treatment plan can be derived

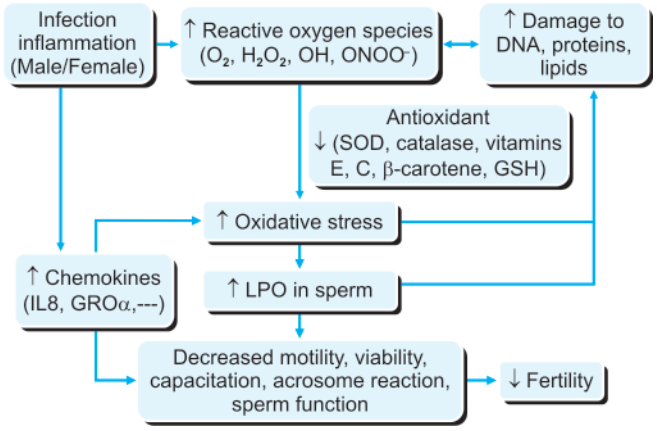

Figure 14.2: Oxidative stress as a result of infection and inflammation can result from two pathways—an increase in reactive oxygen species (ROS) in conjuction with a decrease in antioxidant defense or an increase in chemokines. Both pathways can lead to lipid peroxidation of the sperm plasma membrane, and thus, sperm dysfunction. Ultimately, oxidative stress can result in male infertility

in ROS production by sperm. During maturation and differentiation, spermatocytes lose their cytoplasm in order to transform into mature spermatids (Agarwal, Nallella et al 2004). If residual cytoplasm is retained, it is called a cytoplasmic droplet and is considered immature and defective (Figure 14.3). It is considered that these defective sperm with cytoplasmic droplets are able to produce excess ROS (Aziz, Saleh et al 2004). Aiken et al (1992), postulated that ROS production may be generated in two ways: through the membrane in the NADPH-oxidase system, and through the NADH-dependant oxidoreductase of mitochondria. Even though mitochondria have been implicated in ROS production, mitochondrial DNA is shown to be adversely effected by excess ROS. Sperm have abundant mitochondria due to their need for constant energy during motility. Evenson et al (1982) states that defective mitochondria play a major role in ROS production and in turn effects sperm function, especially motility. Sperm with ROS induced DNA damage lose their ability to fertilize an oocyte (Agarwal and Prabakaran 2005). Not only is mitochondrial DNA affected by ROS species but sperm DNA is adversely effected. An experiment by Alvarez et al (2002) concluded that leukocytospermic patients had semen samples with a significant increase in DNA damage when compared to non-leukocytospermic samples. Alvarez et al (2002) also found that leukocytospermia was directly correlated with an increase in immature cell lines. Induction of DNA damage can also lead to apoptosis or programmed cell

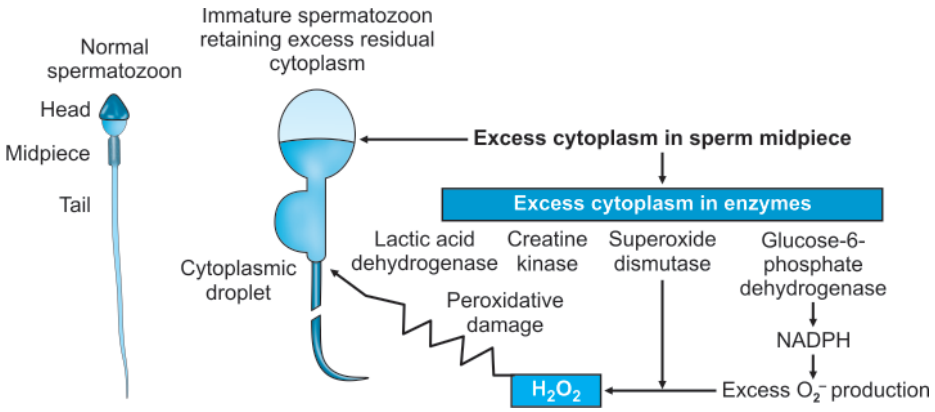

Figure 14.3: Cytoplasmic enzymes are extruded during the final stages of spermiogenesis. If the cytoplasmic enzymes are not removed, then oxidative stress is induced, leading to peroxidative damage to the sperm

death. Apoptosis is a physiological mechanism that ensures that damaged or defective cells do not survive. Mitochondria play a significant role in apoptosis through cytochrome *C* and activation of caspases (Wang et al 2003; Agarwal and Prabakaran 2005). It has been shown that high levels of cytochrome *C* in seminal plasma indicates ROS damage of mitochondria. Higher levels of ROS have also shown to increase the rate of apoptosis (Wang, Sharma et al 2003). It was determined by Moustafa et al (Moustafa, Sharma et al 2004) that infertile men had higher levels of ROS and percentages of apoptosis than fertile men.

Etiology of Leukocytospermia

There are many known reasons for the presence of leukocytes in the seminal fluid. Urinary tract infections and sexually transmitted diseases are common causes for increased leukocyte count. As discussed earlier, peroxidase-positive leukocytes such as macrophages and neutrophils are the main immunosurveillance leukocytes. Chemotaxtic cytokines such as interleukins, interferons, and tumor necrosis factors are generated by infected and inflamed cells and lead to peroxidase-positive leukocyte migration and infiltration within the seminal plasma (Shimoya, Matsuzaki et al 1993).

Bacterial and Viral Etiology

Bacterial prostatitis is one of the main culprits for the increased migration of leukocytes into the genitourinary tract. It is estimated that 50% of men will experience an acute prostate infection during their lifetime with 10% becoming chronic conditions (Schaeffer 2003). Whether acute or chronic, most prostate infections are a result of either another urinary tract infection or sexually transmitted diseases (Fraczek and Kurpisz 2007). All types of bacteria including gram-positive, gram-negative, and atypical bacteria will cause an influx of leukocytes and increase in ROS (Mazzilli et al 1994). Spinal cord injury patients have also been shown to have an increased leukocyte count in their semen due to an increased susceptibility to urinary tract pathologies (Padron, Brackett et al 1997). It has also been postulated that viral infection might play a role in leukocytospermia. Kapranos et al (2003) found that patients with herpes simplex virus had a 10-fold increase in the rate of leukocytospermia. Similarly, systemic viral infections have been linked with increased number of leukocytes in semen. Human immunodeficiency virus (HIV) was found to cause an increased leukocyte count in semen as compared to a control (Umapathy, Simbini et al 2001). Hepatitis B and C patients have been shown to have impaired sperm motility but whether it is caused by increased leukocytes and OS in semen is still under investigation (Durazzo, Premoli et al 2006).

Abacterial Etiology

One of the more elusive causes of leukocytospermia is autoimmune and inflammatory processes in the absence of bacteria. Pasqualotto et al (2000) stated that chronic abacterial autoimmune prostatitis has been linked to considerably elevated levels of ROS from increased levels of leukocytes. As discussed earlier it is believed that an increase and release in pro-inflammatory cytokines such as interleukins, interferon, and tumor necrosis factors

leads to increased chemotaxis and activation of leukocytes which results in OS (Motrich, Maccioni et al 2005). Further investigation into the exact mechanism of autoimmune response is ongoing; although, one theory finds that a polymorphism of cytokine links to autoimmune prostatitis if cytokine IL-10 may be responsible (Shoskes, Albakri et al 2002).

Vasectomy Reversal

It has been proposed that vasectomy reversal leads to an increase in OS and seminal leukocyte count. It is hypothesized that disruption to the blood-testis barrier leads to an increased number and activation of pro-inflammatory cytokines (Shapiro, Muller et al 1998; Filippini et al 2001).

Lifestyle Factors

Smoking has been explored and is hypothesized to be linked to an increase in seminal leukocytes. Saleh et al (2002) reported that there was a 48% increase in seminal leukocytes and a 107% increase in seminal ROS levels among smokers when compared to a control. This study was in response to research by Fraga et al (1996), where a significant increase in levels of a nicotine by product (8-OHdG) in the seminal fluid of smokers was found.

Obesity has also been reviewed as a factor with deleterious effects on semen parameters. Singer et al (2007) stated that adipose tissue releases cytokines and other pro-inflammatory mediators, which in turn causes oxidative stress and leukocyte migration. Heating in the groin by excess adipose tissue may affect the testicles and lead to increased OS (Banks, King et al 2005).

SELF DEFENSE MECHANISMS, DETECTION AND TREATMENT

Self Defense Mechanisms

The seminal fluid naturally possesses anti-oxidant enzymes to combat oxidative stress. These enzymes play an essential role in replacing the cytoplasmic enzymes lost by sperm during spermiogenesis. In addition, seminal fluid also contains non-enzyme anti-oxidants, such as vitamins C and E, pyruvate, glutathione and carnitine. Some authors reported that aging, diet, smoking and lifestyle tend to reduce these innate immune defenses, lowering semen quality and impairing fertilizing capacity.

Non-enzymatic Antioxidants

Vitamin E

In vitro studies demonstrate that vitamin E can provide a protective role for cryopreserved sperm. Assisted reproductive technique procedures including cryopreservation and sperm preparation are associated with increased OS, resulting in detrimental effects to sperm motility and integrity (Agarwal et al 2006). Vitamin E, which includes the tocopherol compounds, is a major antioxidant that binds to the products of the lipid peroxidation chain reaction, thereby inhibitng further propagation of the oxidation reaction. Vitamin E appears to have a dose-dependent protective effect on the sperm membrane (Hull et al 2000). One study found that by adding vitamin E to cryoprotectants at a dose of 10 mmol/liter the effects on sperm motility by OS can be reduced (Hull et al 2000). Another study was performed on asthenozoospermic patients who received oral vitamin E (300 mg/day). The malondialdehyde (MDA) concentration, a marker for lipid peroxidation, in spermatozoa significantly decreased and sperm motility improved (Suleiman et al 1996). Compared to the placebo treated patients, which reported no pregnancies, eleven of the 52 treated patients (21%) impregnated their wives, with nine of the women successfully giving birth.

While vitamin E supplementation leads to significantly improved sperm motility and decreased MDA concentrations, insignificant improvements in sperm quality and quantity were observed in studies treating patients with doses of vitamin E below 400 mg (Geva et al 1996).

Vitamin C

Vitamin C (ascorbate) is another chain-breaking antioxidant that can help lower oxidative stress. Accounting for up to 65% of the antioxidant capacity of the seminal plasma, vitamin C concentration is much more prevalent in seminal plasma than blood plasma (364 µmol/liter to 40 µmol/liter) (Lewis et al 1997). Evidence indicates a dose-dependent effect of vitamin C on sperm motility. Dawson et al (1992) demonstrated that improvements in sperm motility occurred in 200 mg and 1,000 mg vitamin C supplemented groups, with the 1,000 mg group exhibiting the highest improvements. The third group, a placebo supplemented group, showed no improvement in sperm quality.

Not only can vitamin C improve sperm motility parameters, vitamin C can also play a role in regards to oxidative damage to DNA. Fraga et al (1991) discovered a correlation between seminal fluid vitamin C levels and DNA damage. Using semen samples from healthy donors, Fraga et al reported that high levels of 8-OHdG (a biomarker of DNA damage) were correlated with low levels of seminal vitamin C. Varying the vitamin C intake in another group found that seminal vitamin C levels and 8-OHdG levels were directly correlated with intake levels. When vitamin C intake was decreased from 250 mg/day to 5 mg/day, a decrease in seminal vitamin C levels was found along with a significant increase in 8-OHdG levels. Similarly, repletion of vitamin C intake resulted in increased seminal Vitamin C levels and decreased 8-OHdG levels in sperm DNA.

Glutathione

Glutathione is present in concentrations of 0.5-10 mmol/liter and is the most abundant non-thiol protein in mammalian cells (Irvine 1996). Glutathione acts as a protectant with its nucleophilic sulphydryl group (SH) and attenuates oxidative stress from oxidants and electrophiles, such as superoxide and O_2. Glutathione is also important for maintaining sperm motility. A study suggests that glutathione deficiency can lead to instability of the midpiece, causing impaired sperm motility (Hansen et al 1996). Lenzi et al (2004) found that treatment with glutathione of infertile men with unilateral varicocele or genital tract inflammation led to significant sperm quality improvement. This is significant because varicoceles and genital tract inflammation have been correlated with increased oxidative stress (Pasqualotto et al 2000).

Other Non-enzymatic Antioxidants

Further antioxidant support can be supplied by molecules such as N-acetyl L-cysteine, carotenoids, coenzyme Q10 and carnitines. N-acetyl L-cysteine, a precursor of glutathione, has been shown to reduce ROS production and help improve sperm motility (Oeda et al 1997; Agarwal et al 2004). Carotenoids such as beta-carotene and lycopene can protect the plasma membrane against lipid peroxidation (Gupta and Kumar 2002). Evidence suggests that lycopene, which is found in abundance in tomatoes, can be ten times more potent that vitamin E in binding singlet oxygen and protecting the plasma membrane (Di Mascio et al 1989). Coenzyme Q10 is found in the sperm mid-piece (Lewin and Lavon 1997) and has been demonstrated to inhibit H_2O_2 formation in both seminal fluid and seminal plasma (Alleva et al 1997). In contrast, carnitines are dietary antioxidants that improve sperm motility and remove excess intracellular toxic acetyl-CoA, which is a substrate for mitochondrial ROS production (Agarwal and Said 2004).

Enzymatic Antioxidants

Superoxide Dismutase

Superoxide dismutase (SOD) is considered a scavenging antioxidant that acts against both sperm-intracellular and extracellular ROS (Kovalski et al 1992). SOD protects the spermatozoa from lipid peroxidation of the plasma membrane by superoxide anion. By conjugating with catalse or glutathione peroxidase, SOD can also deter H_2O_2 action. Kobayashi et al (1991) reported that the addition of exogenous SOD (400 IU/ml) to the sperm suspension led to a significant reduction in loss of motility and MDA concentration. SOD also has the capability to prevent premature hyperactivation and capacitation, since superoxide radicals can induce hyperactivation and capacitation before ejaculation.

Glutathione Peroxidase/Reductase System

Glutathione combines with vitamin E and selenium to form glutathione peroxidase. The glutathione peroxidase/reductase system prevents both lipid peroxidation initiation and chain reaction propagation by scavenging both H_2O_2 and lipid peroxides. Glutathione reductase catalyzes the reduction glutathione disulfide to glutathione, which provides a protectant against OS.

Catalase

Catalase is responsible for the neutralization of intracellular and extracellular H_2O_2. Catalse catalyzes the breakdown of H_2O_2 to water and oxygen. Studies have demonstrated that catalase can reduce the loss of motility in addition to reducing lipid peroxidation and DNA damage (Kovalski et al 1992; Lopes et al 1998). Catalase can also activate capacitation through NO-induced sperm capacitation (Lamirande and Gagnon 1995).

Detection

Leukocytes

Leukocytes can be counted manually and microscopically, but doing so is unreliable when counting low numbers of white blood cells (WBC) in semen because a sampling error can occur. In addition, inaccurate numbers will likely occur from the similar morphological characteristics between some WBC and immature germ cells. Described below are more accurate methods of determining the leukocyte concentration.

Endtz Test

In 1974, Endtz et al described a peroxidase test that could detect granulocytes. Using myeloperoxidase staining, the leukocyte concentration in semen can be determined. The Endtz test has the advantage of being relatively easy to perform, but it cannot detect polymorphs and other white blood cells, such as degranulated neutrophils. To assess the role of leukocytes in sperm dysfunction, differentiation between the subtypes of WBC is essential; thus, the Endtz test, while efficient and cost effective, may not be the most adequate test for leukocyte concentration.

Polymorphonuclear (PMN) Granulocyte-elastase Detection

A commercial immunoassay (Ecoline PMN-Elastase; Merck, Milan, Italy) is used to indirectly determine the PMN elastase concentrations by photometrically measuring the turbidity of a latex and human PMN elastase solution. PMN elastase concentration has been reported to be a marker for measuring leukocytospermia (Wolff and Anderson 1988).

Immunocytochemistry

Immunohistological staining is considered the gold standard of semen WBC tests and involves monoclonal antibodies that target all specific WBC subpopulations (Eggert-Kruse et al 1992; Kiessling et al 1993). The monoclonal antibodies used are anti-CD45 and anti-CD-53. Although accurate, immunohistological staining is expensive, time consuming, and not standardized.

Flow Cytometry

Ricci et al (2000) established a new method of combining flow cytometry with monoclonal antibodies to measure and evaluate leukocytes in semen. The method uses the same antibodies as immunocytochemistry (anti-CD45 and anti-CD-53) and shows more specificity than both the elastase and Endtz test (Ricci et al 2000).

Reactive Oxygen Species

Chemiluminescence Assay

The chemiluminescence assay is the most commonly used method to measure ROS in semen (Kobayashi et al. 2001). The probes used are luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-*N*-methylacridinum nitrate). Luminol measures both intra- and extracellular seminal ROS radicals- OH^-, H_2O_2 and O_2^-. Lucigenin, on the other hand, only measures extracellular ROS- specifically OH^- and O_2^-. Light sensitive chemical reagents are used in the chemiluminescence assay and the luminescence is usually measured by photon counting or current counting luminometers. Results are reported as counted photon per minute (cpm), relative light units (RLU), and millivolts per second (mV/s).

Flow Cytometry

Flow cytometry is used to measure ROS in addition to the chemiluminescence assay (Marchetti et al 2002). Using a low number of cells, identification of individual intracellular ROS radicals can be obtained. Common probes are 2', 7'-dichlorofluorescein-diacetate and hydroethidine, which detect H_2O_2 and O_2^-, respectively.

Other methods to measure ROS can be categorized into 2 categories. They are reactions involving nitroblue tetrazolium (NBT) or cytochrome c-Fe^{3+} complexes and the electron spin resonance methods. Measurement of reactions involving NBT and cytochrome c-Fe^{3+} determine the amount of ROS on the cell membrane surface. The electron spin resonance method is more sensitive and can identify the different types of ROS generated within the cell, but it is very inefficient and costly (Sikka et al 1995).

Indirect Method

The stable end-products of the peroxidative reaction can be used to determine ROS levels as well. Malondialdehyde (MDA) is one such product and can be estimated with the thiobarbituric acid assay (Alvarez et al 1987). In addition, peroxidative damage can be measured by levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), which is an end-product of DNA damage by OS (Agarwal and Said 2003).

Oxidative Stress

Reactive oxygen species–total antioxidant capacity score ROS and total antioxidant capacity (TAC) vary between individuals, and since OS is based on the difference between the amount of ROS and the physiological defense of the individual, measuring either ROS or TAC alone is not adequate enough to determine OS. In order to accommodate for the variations, a composite score, based on a statistical formula, has been developed by Sharma et al (1999) to assess OS. Described as the ROS-TAC score, this composite score is proposed by Sharma et al as a novel way to measure OS and predict male infertility. Fertile men tend to have high ROS-TAC scores whereas infertile men generally have lower (<30) ROS-TAC scores (Sharma et al 1999).

Measurement of OS in Neat Semen

In addition to measuring ROS, chemiluminescence assays can be used to determine oxidative stress (Saleh et al 2001). By measuring ROS levels directly in neat semen, infertile men were reliably classified into OS-positive and OS-negative groups. Saleh et al suggested that measuring OS in this manner provided a diagnostic and prognostic value similar to that of the ROS-TAC scores obtained from washed semen.

Clinical Treatment

Antioxidant Supplementation

As discussed previously, *in vitro* studies have demonstrated the positive effects of exogenous introduction of anti-oxidants such as SOD (Kobayashi et al 1991). Whether or not that translates into treatment success for leukocytospermic patients is still debated. Dietary antioxidant supplementation has provided some positive results. In asthenoteratospermic men with leukocytospermia, Piomboni et al (2008) showed the anti-oxidant effects of beta glucan, papaya, lactoferrin, vitamin C, and vitamin E on sperm characteristics. After treatment for 90 days, positive effects on percentage of morphologically normal sperm and total progressive motility were reported. In addition, leukocyte concentration was significantly diminished and chromatin integrity improved. Another study supported the notion that antioxidant supplementation is beneficial to leukocytospermic infertile men. A study indicated that leukocyte concentrations decreased and total antioxidant power increased after antioxidant and antibiotic treatment (Yadav et al 2006). Viable sperm were also reported to be increased after treatment.

Non-steroidal Anti-inflammatory Drugs (NSAIDs)

Anti-inflammatory drugs of different types have been found effective in the treatment of asymptomatic and symptomatic leukocytospermia. The principal mechanism of action for NSAIDs is through cyclooxygenase (COX) inhibition. COX exists in two isoforms: COX-1 and COX-2, and NSAIDs specifically inhibit COX-2 (Bolten 1998). A study investigated the effect of a COX-2 inhibitor on leukocyte concentrations in infertile males with abacterial leukocytospermia (Gambera et al 2007). Gambera et al found that one month therapy with a daily dose of 25 mg of rofecoxib was enough to yield a significant decrease in leukocyte concentrations. An improvement in sperm motility and morphology was found as well.

In addition, NSAIDs have been found to help reduce ROS levels in leukocytospermic patients (Vicari et al 2002), when used in conjunction with carnitines, as long as the supplementation timing is not concomitant. After three months of NSAID therapy, administration of carnitine yielded a significant decrease in ROS production. As promising as the studies have been thus far, the direct effect of NSAIDs on sperm quality and the outcome of assisted reproductive procedures still have to be investigated.

Antibiotics

The significance of antibiotic treatment is supported in a study by Skau and Folstad (2003). Without diagnosed genital tract infections, leukocytospermic men were treated with antibiotics. The results showed a significant improvement in ejaculate volume, sperm concentration, percentage of motile spermatozoa, and percentage of spermatozoa with normal morphology. The leukocyte concentration was also significantly reduced. These results indicate the importance of using broad-spectrum antibiotics to treat both leukocytospermic men and men with bacterial infections.

CONCLUSION

As the role of ROS and OS in male infertility becomes clearer, the determination and differentiation of the source for ROS has to be elucidated. Unfortunately, controversy still exists when discussing the source of ROS

in semen. Strong evidence can be found for both germ cells and leukocytes as the source for ROS. However, many studies have reported that leukocytospermia is correlated with poor semen quality, thus, possibly linking it as a cause for male infertility. On the other hand, the varying numbers of WBC in infertile patients indicate that other factors may be responsible for the poor semen quality.

The possibility of sperm preparation procedures during assisted reproductive techniques to magnify the effects of leukocytospermia further complicates the situation. Because sperm washing techniques eliminate the natural antioxidant defense systems of the seminal fluid, the effects of ROS production by leukocytes induce a significant increase in OS on the sperm. Therefore, exogenous introduction of antioxidants during ART procedures may be a solution. However, a consensus and standard on the type and dosage of antioxidants to be used still needs to be established. Perhaps, antioxidant treatment is beneficial to only infertile leukocytospermic men and may not be beneficial for other forms of OS induced infertility, such as varicoceles.

Unfortunately though, evaluation of OS status and use of antioxidants is not universal and varies from clinic to clinic. Further simplification and standardization of ROS and OS measuring techniques need to be done before the benefits of antioxidant treatment can truly be determined. With the increase in use of ART procedures as a means to bypass male infertility issues, maximizing the efficiency of the procedures is essential. Thus, with the presence of leukocytes in almost every semen sample, elucidating the role of leukocytospermia could play a beneficial role in treating male infertility treatment.

BIBLIOGRAPHY

1. Agarwal A, Allamaneni SS, Nallella KP, George AT, Mascha E. Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis. Fertil Steril 2005;84(1):228-31.
2. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol 2008;59(1):2-11.
3. Agarwal A, Nallella KP, Allamaneni SS, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online 2004;8(6):616-27.
4. Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 2005;43(11):963-74.
5. Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG. Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 2006;86(3):503-12.
6. Agarwal A, Said TM. Carnitines and male infertility. Reprod Biomed Online 2004;8(4): 376-84.
7. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 2003;79(4):829-43.
8. Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am 2002;29(4):817-27.
9. Aitken RJ, Buckingham DW, West KM. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J Cell Physiol 1992;151(3):466-77.
10. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 1989;41(1):183-97.
11. Alleva R, Scararmucci A, Mantero F, Bompadre S, Leoni L, Littarru GP. The protective role of ubiquinol-10 against formation of lipid hydroperoxides in human seminal fluid. Mol Aspects Med 1997;18 Suppl:S221-8.
12. Alvarez JG, Sharma RK, Ollero M, et al. Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. Fertil Steril 2002;78(2):319-29.
13. Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl 1987;8(5):338-48.
14. Alverez K, Haswell C, St Clair M, et al. Sequences of the mouse N-acetylglucosaminyltransferase V (Mgat5) mRNA and an mRNA expressed by an Mgat5-deficient cell line. Glycobiology 2002;12(7):389-94.
15. Aziz N, Agarwal A, Lewis-Jones I, Sharma RK, Thomas AJ, Jr. Novel associations between specific sperm morphological defects and leukocytospermia. Fertil Steril 2004;82(3):621-27.
16. Aziz N, Saleh RA, Sharma RK, et al. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril 2004;81(2):349-54.
17. Banks S, King SA, Irvine DS, Saunders PT. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. Reproduction 2005;129(4):505-14.
18. Bolten WW. Scientific rationale for specific inhibition of COX-2. J Rheumatol Suppl 1998;51:2-7.
19. Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. Fertil Steril 1992;58(5):1034-39.
20. de Lamirande E, Gagnon C. Capacitation-associated production of superoxide anion by human spermatozoa. Free Radic Biol Med 1995;18(3):487-95.
21. de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. Rev Reprod 1997;2(1):48-54.

22. de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997;3(3):175-94.
23. Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274(2):532-8.
24. Durazzo M, Premoli A, Di Bisceglie C, et al. Alterations of seminal and hormonal parameters: An extrahepatic manifestation of HCV infection? World J Gastroenterol 2006;12(19):3073-76.
25. Eggert-Kruse W, Bellmann A, Rohr G, Tilgen W, Runnebaum B. Differentiation of round cells in semen by means of monoclonal antibodies and relationship with male fertility. Fertil Steril 1992;58(5):1046-55.
26. Endtz AW. A rapid staining method for differentiating granulocytes from "germinal cells" in Papanicolaou-stained semen. Acta Cytol 1974;18(1):2-7.
27. Erenpreiss J, Hlevicka S, Zalkalns J, Erenpreisa J. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. J Androl 2002;23(5):717-23.
28. Evenson DP, Darzynkiewicz Z, Melamed MR. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem 1982;30(3):279-80.
29. Filippini A, Riccioli A, Padula F, et al. Control and impairment of immune privilege in the testis and in semen. Hum Reprod Update 2001;7(5):444-9.
30. Fraczek M, Kurpisz M. Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. J Androl 2007;28(2):325-33.
31. Fraczek M, Sanocka D, Kamieniczna M, Kurpisz M. Proinflammatory cytokines as an intermediate factor enhancing lipid sperm membrane peroxidation in in vitro conditions. J Androl 2008;29(1):85-92.
32. Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci USA 1991;88(24):11003-6.
33. Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. Mutat Res 1996;351(2):199-203.
34. Gambera L, Serafini F, Morgante G, Focarelli R, De Leo V, Piomboni P. Sperm quality and pregnancy rate after COX-2 inhibitor therapy of infertile males with abacterial leukocytospermia. Hum Reprod 2007;22(4):1047-51.
35. Gavella M, Lipovac V. NADH-dependent oxidoreductase (diaphorase) activity and isozyme pattern of sperm in infertile men. Arch Androl 1992; 28(2):135-41.
36. Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. Int J Androl 1998;21(2):81-94.
37. Griveau JF, Le Lannou D. Influence of oxygen tension on reactive oxygen species production and human sperm function. Int J Androl 1997;20(4):195-200.
38. Griveau JF, Le Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. Int J Androl 1997;20(2):61-9.
39. Gupta NP, Kumar R. Lycopene therapy in idiopathic male infertility—a preliminary report. Int Urol Nephrol 2002;34(3):369-72.
40. Hansen JC, Deguchi Y. Selenium and fertility in animals and man—a review. Acta Vet Scand 1996;37(1):19-30.
41. Hull MG, North K, Taylor H, Farrow A, Ford WC. Delayed conception and active and passive smoking. The Avon Longitudinal Study of Pregnancy and Childhood Study Team. Fertil Steril 2000;74(4):725-33.
42. Irvine DS. Glutathione as a treatment for male infertility. Rev Reprod 1996;1(1):6-12.
43. Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R. Catalase activity in human spermatozoa and seminal plasma. Gamete Res 1989;24(2):185-96.
44. Kapranos N, Petrakou E, Anastasiadou C, Kotronias D. Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic. Fertil Steril 2003;79 Suppl 3:1566-70.
45. Kiessling AA, Yin HZ, Purohit A, Kowal M, Wolf B. Formaldehyde-fixed semen is suitable and safer for leukocyte detection and DNA amplification. Fertil Steril 1993;60(3):576-81.
46. Kobayashi H, Gil-Guzman E, Mahran AM, et al. Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. J Androl 2001;22(4):568-74.
47. Kobayashi T, Miyazaki T, Natori M, Nozawa S. Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. Hum Reprod 1991;6(7):987-91.
48. Kovalski NN, de Lamirande E, Gagnon C. Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effect of seminal plasma and scavengers. Fertil Steril 1992;58(4):809-16.
49. Lackner JE, Lakovic E, Waldhor T, Schatzl G, Marberger M. Spontaneous variation of leukocytospermia in asymptomatic infertile males. Fertil Steril 2008;90(5):1757-60.
50. Lenzi A, Sgro P, Salacone P et al. A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. Fertil Steril 2004;81(6):1578-84.
51. Lewin A, Lavon H. The effect of coenzyme Q10 on sperm motility and function. Mol Aspects Med 1997;18 Suppl:S213-9.
52. Lewis SE, Sterling ES, Young IS, Thompson W. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 1997;67(1):142-7.
53. Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 1998;13(4):896-900.
54. Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 2002;17(5):1257-65.
55. Mazzilli F, Rossi T, Marchesini M, Ronconi C, Dondero F. Superoxide anion in human semen related to seminal

parameters and clinical aspects. Fertil Steril 1994; 62(4):862-8.
56. Moskovtsev SI, Willis J, White J, Mullen JB. Leukocytospermia: Relationship to sperm deoxyribonucleic acid integrity in patients evaluated for male factor infertility. Fertil Steril 2007;88(3):737-40.
57. Motrich RD, Maccioni M, Molina R, et al. Reduced semen quality in chronic prostatitis patients that have cellular autoimmune response to prostate antigens. Hum Reprod 2005;20(9):2567-72.
58. Moustafa MH, Sharma RK, Thornton J, et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod 2004;19(1):129-38.
59. Oeda T, Henkel R, Ohmori H, Schill WB. Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: A possible therapeutic modality for male factor infertility? Andrologia 1997;29(3):125-31.
60. Ohyashiki JH, Ohyashiki K, Shimizu H, et al. Testicular tumor as the first manifestation of B-lymphoid blastic crisis in a case of Ph-positive chronic myelogenous leukemia. Am J Hematol 1988;29(3):164-7.
61. Padron OF, Brackett NL, Sharma RK, Lynne CM, Thomas AJ, Jr., Agarwal A. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. Fertil Steril 1997;67(6):1115-20.
62. Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil Steril 2000;73(3):459-64.
63. Pasqualotto FF, Sharma RK, Potts JM, Nelson DR, Thomas AJ, Agarwal A. Seminal oxidative stress in patients with chronic prostatitis. Urology 2000;55(6):881-5.
64. Piomboni P, Gambera L, Serafini F, Campanella G, Morgante G, De Leo V. Sperm quality improvement after natural anti-oxidant treatment of asthenoteratospermic men with leukocytospermia. Asian J Androl 2008; 10(2):201-6.
65. Ricci G, Presani G, Guaschino S, Simeone R, Perticarari S. Leukocyte detection in human semen using flow cytometry. Hum Reprod 2000;15(6):1329-37.
66. Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ, Jr. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil Steril 2002;78(3):491-9.
67. Saleh RA, Esfandiari N, Sharma RK, Nelson DR, Thomas AJ. Diagnostic and prognostic value of measurement of reactive oxygen species in neat semen [abstract O-24]. In: Programs and Abstracts of the 57th Annual Meeting of the American Society for Reproductive Medicine. Orlando (FL); 2001:9.
68. Schaeffer AJ. Epidemiology and demographics of prostatitis. Andrologia 2003;35(5):252-7.
69. Seronello S, Sheikh MY, Choi J. Redox regulation of hepatitis C in nonalcoholic and alcoholic liver. Free Radic Biol Med 2007;43(6):869-82.
70. Shapiro RH, Muller CH, Chen G, Berger RE. Vasectomy reversal associated with increased reactive oxygen species production by seminal fluid leukocytes and sperm. J Urol 1998;160(4):1341-46.
71. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. Urology 1996;48(6):835-50.
72. Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ, Jr., Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J Androl 2001;22(4):575-83.
73. Shimoya K, Matsuzaki N, Tsutsui T, Taniguchi T, Saji F, Tanizawa O. Detection of interleukin-8 (IL-8) in seminal plasma and elevated IL-8 in seminal plasma of infertile patients with leukospermia. Fertil Steril 1993;59(4):885-8.
74. Shoskes DA, Albakri Q, Thomas K, Cook D. Cytokine polymorphisms in men with chronic prostatitis/chronic pelvic pain syndrome: association with diagnosis and treatment response. J Urol 2002;168(1):331-5.
75. Singer G, Granger DN. Inflammatory responses underlying the microvascular dysfunction associated with obesity and insulin resistance. Microcirculation 2007;14(4-5):375-87.
76. Skau PA, Folstad I. Do bacterial infections cause reduced ejaculate quality? A meta-analysis of antibiotic treatment of male infertility. Behavioral Ecology 2003;14(1):40–7.
77. Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: Protective role of vitamin E. J Androl 1996;17(5):530-37.
78. Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 1998;13(6):1429-36.
79. Umapathy E, Simbini T, Chipata T, Mbizvo M. Sperm characteristics and accessory sex gland functions in HIV-infected men. Arch Androl 2001;46(2):153-8.
80. Vicari E, La Vignera S, Calogero AE. Antioxidant treatment with carnitines is effective in infertile patients with prostatovesiculoepididymitis and elevated seminal leukocyte concentrations after treatment with nonsteroidal anti-inflammatory compounds. Fertil Steril 2002;78(6):1203-8.
81. Wang X, Sharma RK, Sikka SC, Thomas AJ, Jr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril 2003;80(3):531-5.
82. Wolff H, Anderson DJ. Evaluation of granulocyte elastase as a seminal plasma marker for leukocytospermia. Fertil Steril 1988;50(1):129-32.
83. Wolff H. The biologic significance of white blood cells in semen. Fertil Steril 1995;63(6):1143-57.
84. Yadav SB, Suryakar AN, Huddedar AD, Shukla PS. Effect of antioxidants and antibiotics on levels of seminal oxidative stress in leukocytospermic infertile men. Indian Journal of Clinical Biochemistry 2006;21(1):152-6.

CHAPTER 15

Genetics of Male Infertility

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ABSTRACT

Infertility is a health problem of global concern. One in 7 couples experience infertility and subfertility. Male and female factor each account for about 40% of the cases of infertility and in 20% of the cases it is a combination. This chapter is an overview of cytogenetic and molecular causes of infertility and disorders of sexual differentiation. Also protocols for cytogenetic and molecular analysis in diagnostic workup of an infertile male are given at the end of the chapter.

INTRODUCTION

Infertility affects 12 to 15% of couples attempting pregnancy. A World Health Organization (WHO) multicentric study (1987) reported that male factor is an important contributory cause in about half of these cases. 30 to 40% of men in reproductive age group have a qualitative or quantitative defect in sperm production.[1] Spermatogenesis is especially sensitive to the balance between many cellular processes, and genetic abnormalities and mutations in several genes may lead to partial or complete spermatogenic arrest and result in oligozoospermia/azoospermia. Genetic aberrations account for a significant fraction of these cases and can be chromosomal, monogenic or multifactorial and affect not only gamete production but also the ability of gametes fertilization capability and embryonic development. The management of infertile couples (especially severe male factor infertility) using assisted reproductive technology (ART) has increased the importance of understanding the genetic etiology of infertility. The presence of a genetic abnormality determines the prognosis and aids in providing comprehensive counseling and the most adapted therapeutics to the infertile couple. Although ART has offered hope to millions of infertile couples, it also has increased the risk of transmission of genetic anomalies to the offspring with a concomitant increase in incidence of both major and minor congenital malformations.

In 1993, WHO recommended a standardized approach to the investigation and diagnosis of infertile couples.[2] It established 16 diagnostic categories for men, including acquired, iatrogenic and idiopathic. Although several sex chromosomal and autosomal genes regulate normal reproductive functioning, this chapter highlights some of the important chromosomal/genetic causes of infertility and also the protocols for blood culture, cytogenetic analysis and polymerase chain reaction (PCR)-based Y chromosome microdeletion screening and methylation-specific PCR (following bisulphate modification).

Chromosomal Abnormalities

Chromosomal abnormalities may be numerical or structural and involve either sex chromosomes or autosomes. Although the frequency of chromosomal abnormalities varies among different studies as a result of varying inclusion criteria, several studies have documented that the frequency of chromosomal abnormalities increases with decline in semen quality.[3-6] The frequency of chromosomal abnormalities in idiopathic azoospermic men is 17 to 24%; in oligozoospermic men it is 5 to 10%, about 22 times higher

than in the general population.[3,7] Sex chromosomal abnormalities are predominant in nonobstructive azoospermic men (93%), whereas autosomal structural abnormalities predominate in oligozoospermic cases (67%).[3,8]

Klinefelter syndrome (KFS) is the most common genetic cause of infertility. The 47, XXY chromosomal complement is found more frequently among infertile men (3.5 to 22.6%) than in newborn males (0.07%) and is the most common cause of hypogonadism. These men may be mosaic with multiple cell lines with marked variation in phenotype.[4]

Robertsonian translocations involving acrocentric chromosomes are the most frequent structural autosomal rearrangement found in infertile oligozoospermic men. Vas Assche et al (1996)[6] reported that 0.7% of infertile men present with this abnormality, a nine-fold greater frequency than in newborns. However, in a tertiary referral hospital setting, Kumar et al (2007) reported a higher frequency (3.5%) of Robertsonian translocations in infertile men.[9] Robertsonian translocation carriers may not show any phenotypic abnormality, but they may be infertile. Therefore, in a couple in which a partner is a carrier of a Robertsonian translocation, the couple experiences recurrent spontaneous abortions and usually present with a poor obstetric history.

Reciprocal translocations are reported in 0.5% infertile men as compared with 0.1% newborn infants. These translocations can lead to reduced fertility and spontaneous abortions due to a position effect and depending on the chromosomes involved and the nature of the translocation. These translocations can lead to infertility in a number of ways. For example, by the formation of a quadrivalent that can impede the meiotic process. Alternatively, disjunction can produce genetically unbalanced gametes, asynaptic regions within the pairing cross can lead to meiotic failure and loss of germ cells, or certain translocated segments can undergo aberrant recombination with sex chromosomes and interfere with X chromosome inactivation to cause a lethal gene dosage effect.[10,11]

In cases with Robertsonian translocations, the heterochromatic short p arm of acrocentric chromosomes is lost. This p arm carries the nucleolar organizing region (NOR) for rRNA synthesis and also is associated with the sex vesicle. Thus, p arm loss results in loss of the NOR and can increase the likelihood of cell disruption and germ cell death. Chromosomal inversions also result in reduced recombination and unbalanced gametes, which severely compromise fertility.

Chromosomal Abnormalities in Spermatozoa

Poor semen parameters usually are associated with a high proportion of aneuploid spermatozoa with extra or missing chromosomes. High aneuploidy rate (about 70%) in spermatozoa of infertile men was reported by Calogero et al (2001)[12] and Anton et al (2004).[13] This increased sperm aneuploidy may be associated with paternal age, lifestyle factors (alcohol and nicotine intake) or may be iatrogenic (due to chemotherapeutic regimen). XY-disomy in oligozoo-spermic men may be due to reduced recombination. Egozcue et al (2003)[14] found that the mean incidence of disomy in cytogenetically normal men was about 6.7%, where incidence of abnormal spermatozoa was found to be 36% in carriers of Robertsonian translocations and 81% in carriers of reciprocal translocations.

Monogenic Defects

Several single gene disorders are implicated in the pathogenesis of male infertility. Congenital bilateral absence of vas deferens (CBAVD) (OMIM-277180) is characterized by the absence of vasa and obstruction to outflow of spermatozoa. This form of obstructive azoospermia is responsible for 1 to 2% cases of male infertility. CBAVD also is present in most males with cystic fibrosis (CF). CF is the most common autosomal recessive disease in populations of Caucasian origin, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. About 50 to 83% of men with CBAVD have at least one known CFTR mutation. A higher frequency of CFTR mutations has been described in non-CBAVD obstructive azoospermia, bilateral ejaculatory duct obstruction, Young syndrome and in patients with poor sperm quality and low sperm concentration.

Y Chromosome Microdeletions

The Y chromosome, a group G-acrocentric chromosome, is one of the smallest chromosomes of the human genome. The terminal regions of its short and long arms are flanked by the pseudoautosomal region (PAR1 and PAR11), which undergo end-to-end recombination with the X chromosome. However 95% of the Y chromosome is nonrecombining region (NRY) known as male-specific

Y (MSY). Being the only haploid component of the human genome, the Y chromosome tends to accumulate mutations at a rate much higher than any autosome. This is due to lack of recombination and a high number of repetitive elements spread out along its length. It is divided into seven deletion intervals. The sex determining region (SRY gene) is situated on Yp, while the genes critical for spermatogenesis (azoospermia factor-AZF) on Yq deletion interval 5 and 6.

The Y chromosome has two sets of genes. Those genes that have X homologs and ubiquitous expressions are present as single copies, whereas those genes that have testes-specific expression are present in multiple copies (sexually antagonistic genes). This provides the conditions necessary for male-enhancing, female-detrimental mutations or sexually antagonistic mutations to accumulate on the Y chromosome. The first association between spermatogenic failure and underlying genetic cause was demonstrated by Tiepolo and Zuffardi in 1976.[15] On this basis they proposed the existence of a spermatogenic factor AZF on the long arm of the Y chromosome. This is known as AZF because the most severe phenotype associated with its deletion is azoospermia. This locus consists of three nonoverlapping loci (AZF a, b and c), and deletion of each locus results in spermatogenic arrest at different stages of germ cell development and a characteristic phenotype. These genes play a key role in mRNA processing, transport and splicing, and thus gene deletion results in defects in RNA metabolism and processing. These deletions are specific for spermatogenic failure, and their size and position aid in determining the prognosis and management of infertile cases. AZFa deletions are associated with Sertoli cell-only type I syndrome (SCO type I), AZFb deletions with maturation arrest, and AZFc deletions show a variable phenotype from hypospermatogenesis to SCO type II syndrome.[4,16] AZFc deletions also are associated with progressive decline in sperm count, and thus such cases can be counseled for early sperm cryopreservation.[3] Large AZF deletions make the Y chromosome unstable, and such cases may show a mosaic phenotype 46, XY/45, XO due to loss of the Y chromosome. Children born with such anomaly may have genital ambiguity. The frequency of Yq microdeletions has been reported between 1 and 55%. This large variation is due to inclusion of different patient groups, selection of different numbers and different sets of primers. To establish uniformity of data, the European Academy of Andrology prescribed the use of multiple PCR with seven sets of primers (two for each AZF loci and 1 SRY on Yp as the internal control.[17] Using this set of primers and the same inclusion criteria, the deletion frequency in an Indian idiopathic primary infertile population was found to be 9.83%, similar to that reported in European, Italian and French populations.[3,18] Dada et al (2007), also recommended Yq microdeletion screening from sperm DNA rather than blood because of its greater diagnostic and prognostic significance, especially in men opting for assisted reproduction.[4] They found that sperm harbored a higher frequency of Yq microdeletions and those deletions spanned different AZF loci. Mitra et al (2008) recommended a list of primers that would be ideal for Yq microdeletion screening in the Indian population.[19] Another significant finding reported by Dada et al (2004) was the presence of Yq microdeletions in both idiopathic and nonidiopathic infertile cases (varicocele, cryptorchidism, 47, XXY cases), leading to the necessity to evaluate all these cases for Yq microdeletion.[18] A brief methodology and list of primers for Yq microdeletion screening are provided at the end of this chapter.

Disorders of Sexual Differentiation and Sex Reversal Associated with Male Infertility

Differentiation into male and female is first determined by the genetic sex at the time of fertilization. Male gonadal differentiation begins with expression of the sex-determining region on the Y chromosome (SRY)-(OMIM 480000). SRY is first expressed in mesenchymal cells, which differentiate into Sertoli cells. Sertoli cells express anti-Müllerian hormone (AMH), which is involved in regression of the Müllerian ducts and production of testosterone (T). Testosterone induces formation of internal genitalia, while dihydrotestosterone synthesis from testosterone results in formation of external genitalia (phenotypic sex). Thus structurally, functionally and endocrinologically competent Sertoli cells are required for sexual development and differentiation.

Testicular descent is mediated by InsL 3 produced by Leydig cells.[20,21] Several other genes, such as two zinc finger-containing proteins, steroidogenic factor (SFI) and Wilms' Tumor 1 (WT1), play an important role in commitment and maintenance of indifferent gonads. SFI is also required for optimal secretion of AMH, and T is important for development of hypothalamic pituitary gonadal axis. Mutations in SFI may result in XY sex reversal. WT1 is essential for gonadal and kidney development, and WT1 mutations usually are associated

with several syndromes such as WAGR, Denys Drash and Frasier syndrome and may also lead to development of male pseudohermaphroditism. Aberrant recombination during crossing over may also result in sex reversal, for example, XX males (SRY + ve) or XY males (SRY – ve) or XY individuals with mutant SRY.[22] The prevalence of 46, XX male sex reversal is approximately 1 in 20,000 infertile men (90 times higher than occurs in newborn series). These men usually present with infertility due to absence of AZF.[23] Another gene that is upregulated in XY genital ridges is SOX 9 (SRY - like HMG box). In addition to SOX 9 role in sex determination, it also regulates chondrogenesis.[24] Heterozygous mutations of SOX 9 thus cause campomelic dysplasia, which is associated with XY sex reversal. Huang et al (1999) reported that an extra dose of SOX 9 can initiate testicular differentiation in the absence of SRY in XX females.[25] Another gene, DAX 1 (Dosage sensitive sex adrenal hypoplasia congenital critical region on the X gene), is an antagonist of SRY.[26] This gene can cause dosage-sensitive sex reversal (DSS) in genetic males.[27] This also may explain sex reversal in human genetic males with DAX 1 duplication. Several other genes (on 1p, 9p and 10q) have been reported to be associated with primary sex reversal and infertility in genetic males.

Genetic Syndromes Associated with Male Infertility

Several genetic syndromes are associated with male infertility, e.g. autoimmune polyglandular syndromes type I (OMIM-240300), Beckwith Wiedemann syndrome (OMIM-130650), Prader-Willi syndrome (OMIM-176270) and Noonan syndrome (OMIM-163950). CAG repeat expansion disorders that lead to polyglutamine tract expansion in the androgen receptor gene also has been implicated in male infertility.

Role of Mitochondria in Male Infertility

Human mtDNA is a closed circular extranuclear genome containing 16,569 base pairs. It comprises 37 genes, of which 13 encode for essential components of oxidative phosphorylation (OXPHOS) and ATP synthesis. OXPHOS or the electron transport chain (ETC) consists of 13 subunits, which include complex I (NADH-Q oxido-reductase), seven subunits of complex II (ND1 to ND6 and 4L), complex III (cytochrome b), three subunits of cytochrome oxidase (COX I, COX II and COX III) complex IV, and complex V (ATPase six and eight subunits). Most of the energy required for cell function is generated through oxidative phosphorylation by the ETC.[28,29] Spermatogenesis is a complex process that involves growth and differentiation from germ cell into mature spermatozoa through a unique process. Energy required for spermatogenesis is supplied by the mitochondria. Studies have reported meiotic arrest in cases with mitochondrial defects resulting in hypospermatogenesis. Mitochondria are arranged in a helix of 11 to 13 gyri, with two mitochondria per gyrus that deliver ATP to the axoneme for flagellar movements. mtDNA mutations and irregular mitochondrial arrangement around the mid-piece can impair sperm motility; however, the morphological changes that mitochondria undergo during germ cell differentiation are believed to be influenced by ATP as well.[30] Consequently, any defect in mtDNA and OXPHOS eventually may interfere with the normal differentiation of mitochondria, resulting in defective spermatogenesis. Shamsi et al (2008) reported partially formed microtubular apparatus and absence of the normal 9 + 2 arrangement in infertile asthenozoospermic men who harbored a high number of mitochondrial DNA mutations.[31] Kumar et al (2007) reported a high number of mtDNA nucleotide alterations in cytogenetically normal infertile men with no AZF deletion.[7] These men had high percentage of morphologically abnormal sperm with coiled tails, midpiece defects and also showed ultrastructural defects like irregular mitochondria arrangement around the mid-piece, mitochondria with few cristae, and complete lack of normal microtubular architecture in the sperm axoneme such as displacement of the fibrous sheath and improper arrangement of the doublet microtubule and dynein arms. An average of 1000 mtDNA copies are present in both progressive and nonprogressive spermatozoa of fertile men; the seven-fold increase in mtDNA number that occurs during this process reveals higher ATP requirements for spermatozoa.[30] The arrangement and orientation of mitochondria in the sperm midpiece also are an important factor that supplies energy to the sperm flagella. With a short midpiece, degenerated cristae and the absence of mitochondria, less gyre were observed in infertile patients.[7,32] Pathological changes and defective mitochondrial arrange-ment are believed to affect sperm motility in infertile patients.[31,33] Sperm contain few mtDNA as compared to somatic cells, so phenotypic defects due to mtDNA mutations manifest early and have more deleterious consequences in

spermatozoa. Reactive oxygen species (ROS) produced as a byproduct of OXPHOS induces oxidative stress in their immediate environment and damage mtDNA. Thus, during the course of evolution, majority of mitochondrial genes have translocated to nuclear DNA and only genes regulating OXPHOS are present on mtDNA and these are not transmitted to the offspring. Apart from the mitochondrial genome that encodes mitochondrial proteins for their function, a vast number of nuclear genes also encode for mitochondrial proteins that regulate OXPHOS and also need to be screened.[34] Some of the mutations in nuclear genes such as mitochondrial ribosomal protein S16 (MRPS 16) and EFG1 have been identified in families with defective mitochondrial translation.[35] Supraphysiological ROS levels and mutant mtDNA is one of the issues recently associated with mitochondrial disorders. This is a viscous cycle (Ozawa effect) in which increased ROS production damages mtDNA and its membrane, and damaged mtDNA produced more free radicals. Mitochondrial DNA is not protected by histones, and they are closely associated with the inner mitochondrial membrane where highly mutagenic oxygen radicals are generated as a byproduct of OXPHOS.[36] Therefore, mtDNA is also the first site of damage by free radicals and may be the cause of the accumulation of polymorphisms and mutation in mitochondrial DNA at a rate that is 10 to 17 times faster than occuring in nuclear DNA.[37] Additionally, lack of a repair system and abnormal mitochondrial metabolism may accelerate the rate of accumulation of mitochondrial DNA mutation. Thus, it is important to understand if infertile cases harbor mitochondria or nuclear DNA defects as this has profound diagnostic and prognostic implications.[38]

Epigenetic Alterations in Male Infertility

Each cell of an organism contains exactly the same genome, but only specific subsets of genes are active at a certain time in a particular cell. Gene activity is regulated by the epigenome. The study of processes that are involved in establishing these chromatin modifications is called epigenetics. Genomic imprinting is the phenomenon that causes the differential expression of the two alleles of a gene depending on the parent's sex. Allele-specific expression of certain genes accounts for the requirement for both maternal and paternal genomes in normal development and plays important roles in regulating embryonic growth, placental function and neurobehavioral processes. Genomic imprints are characteristic epigenetic labels that result in functional haploidy at a particular locus, depending on the parental origin of the allele. Thus, monoallelic expression relies on epigenetic mechanisms. DNA methylation of CpG dinucleotides at differentially methylated regions (DMRs) is the best-studied epigenetic mark. Imprint resetting involves erasure of imprints in the primordial germ cells and the acquisition of new sex-specific imprints. Imprinted genes affect prenatal and postnatal growth in greater proportions than do nonimprinted genes.[39,40] Genomic imprinting affects several dozen mammalian genes and results in the expression of those genes from only one of the two parental chromosomes.[41,42] These epigenetic instructions, called imprints, are laid down in the parental germ cells. Methylation marks the imprinted genes differently in egg and sperm, and inheritance of these epigenetic marks is known to lead to differential gene expression.[43,44] All somatic cells have the same set of imprinted genes from both the father and the mother, except for the cells destined to make gametes. A characteristic feature of imprinted genes is that they always are accompanied by other imprinted genes. Aberrant imprinting disturbs development and is the cause of various disease syndromes. For example, in humans the insulin-like growth factor 2 (Igf2) allele inherited from the father (paternal) is expressed. The allele from the mother is not. If both alleles are expressed in a cell, that cell may develop into Wilms' tumor.[45] Imprinted gene defects such as Angelman, Prader-Willi, Beckwith-Wiedemann and Silver-Russell syndromes have been extensively studied in men[46,47] and at least two of these syndromes (Prader-Willi, Beckwith-Wiedemann) are associated with male infertility. Genomic imprinting is reversible, and the imprinted status is reset during gametogenesis. DNA methylation is a mechanism for silencing gene expression and maintaining genome stability. DNA methyl transferase (DNMT3A and DNMT3B) methylate hemimethylated and unmethylated DNA. Mutations of the *de novo* methylase DNMT3A can lead to loss of imprinting and embryonic death, and disruption of DNMT3L in mice has been shown to cause azoospermia in homozygous males. DNA methylation represents a post-synthetic modification. In humans, approximately 1% of all DNA bases are estimated to be 5 mC, commonly known as the fifth base.[48] There are

about 75 imprinted genes which regulate prenatal and neural development. Studies have demonstrated that sperm DNA global methylation is essential for normal embryo development and pregnancy outcome in mice and humans. Aberrant DNA methylation patterns play a major role in carcinogenesis with hypomethylation of oncogenes and hypermethylation of the tumor suppressor gene. Sperm from infertile patients, especially those with oligozoospermia, carry a higher risk of incorrect primary imprints. Recent studies have shown some normal, paternally imprinted genes to be hypomethylated and some paternally imprinted genes to be hypermethylated in the sperm genome. These alterations were specifically found in men with severe oligozoospermia. Sperms with grade D motility harbored more defective methylation patterns as compared with sperms with normal motility (grade A).[49-52] In a recent ongoing study we also found hypomethylation of H19 (a paternally methylated gene) in sperm DNA of oligozoospermic men (unpublished data). In recent decades, semen quality has exhibited a rapid decline, believed to be due to exposure to environmental endocrine disruptors (xenoestrogens) or environmental hormones. These chemicals (persistent organic pollutants) with antiandrogenic effects mimic hormones and influence the cellular programs that determine the methylation pattern. This altered methylation pattern is trapped in the offspring genome and passed on to future generations (transgenerational effects) even when there is no exposure to environmental pollutants and hormonal levels normalize. Thus the current generation may still bear the burden of previous generations' exposure to certain endocrine-disrupting chemicals. This may explain the increased incidence of male genitourinary abnormalities, low sperm count and testicular cancer (hormonal carcinogenesis). Approximately 554 environmentally responsive genes have now been identified. Thus, an individual's phenotype is determined not only by the genotype but also by the epigenotype.

Telomere Length and Telomerase

Telomeres are DNA/protein structures at the ends of chromosomes. Telomere DNA consists of thousands of repeats of TTAGGG that prevent the natural chromosomal ends from being recognized as damaged DNA and consequently protects them from enzymatic modification such as nucleolytic resection and fusion.[53] The terminus part of the telomeres is not blunted but consists of a single-stranded 3′ protrusion of the G-rich strand, called a G-tail or G-overhang. This structure has been observed in yeast, humans, mice, ciliates, plants and trypanosomes.[54,55] In humans, telomere length is maintained from spermatogonia to spermatozoon (i.e. spermatogenesis). DNA polymerase I cannot copy the ends of a DNA strand, and thus, telomere length is maintained by telomerase,[56] a specialized reverse transcriptase complex discovered by Greider and Blackburn in 1985 that counteracts replication-associated telomere shortening.[57] Many studies have addressed the issue of telomere length and telomerase activity in infertile couples. Telomerase has two important components: telomerase reverse transcriptase (TERT) and telomerase RNA. Schrader et al (2000) showed that expression of telomerase subunits and telomerase activity in testicular tissue are highly sensitive and specific markers of gametogenesis in infertile patients.[58] Low-level expression of TERT and telomere RNA in the testis of infertile males is one of the factors for germ cell maturation arrest. Loss of telomerase appears to alter the end structure, rendering the telomere more susceptible to nucleolytic attack and end-to-end fusion. Loss of telomerase RNA in particular leads to defects in cellular differentiation and an increase in genetic instability and infertility. Herrera et al (1999) showed that infertility, aging, immune system defects and other phenotypes appear as a consequence of telomere loss. Telomeric shortening with age may contribute to the loss of sperm viability in men.[59] Telomerase is found only in germ line with some activity in bone marrow and peripheral blood leukocytes. It is present during early development, ensuring that replication associated telomere shortening is suppressed to keep telomere length constant, thereby serving as a biological clock.[60] Telomeres have been observed to shorten in human tissues, including peripheral blood cells, liver, kidney, spleen, dermal fibroblasts and mucosal keratinocytes.[61] However, in most somatic human cells telomerase is not expressed and results in shortening of chromosome ends in such cells every time the cell divides (Harley et al 1990). This process ultimately leads to the generation of too-short telomeres, which have been associated with the process of aging and differentiation.[62] Thus, telomere shortening limits the natural replicative life span of somatic human cells. One study suggests that the telomeric restriction

fragments isolated from DNA from human sperm cells are significantly longer than such fragments isolated from corresponding replicating cells *in vivo*.[63] Telomere shortening is related not only to aging and infertility but also to other physiological genetic phenomenon such as mutations, metabolic disorders and oxidative stress.[60]

Ethical Considerations and Genetic Counseling

Genetic counseling is a communication process whereby individuals and families are educated or told about genetic conditions in their families and about their recurrence risk. Ethics is the branch of philosophy that considers the moral basis for decisions.[64] Ethics and genetics are closely intertwined, as genetic counselors continuously encounter a variety of situations in which ethical principles and guidelines must be consulted and followed. Counselors have several resources at their disposal that provide assistance in working through such ethical dilemmas.

Good counseling depends on accurate diagnosis and involves addressing the psychosocial issues that accompany the diagnosis. The counselor supports the family in learning about the diagnosis and in decision-making about issues surrounding it. Genetic counseling is based on the principles of nondirectiveness and a client-centered approach. During the majority of sessions, the genetic counselor obtains a detailed family, medical and pregnancy history in the form of a pedigree. In addition to providing valuable information about the medical aspects of the family history, the genetic counselor gains useful information about the dynamics of the family in general and in relation to the condition in question.[65] Genetic counselors routinely interact with individuals who have a personal or family history of a genetic syndrome. Certain chromosomal abnormalities and genetic conditions may result in infertility. Therefore, when an individual or couple experiences infertility, possible genetic and cytogenetic causes must be considered and evaluated. If such a cause is identified, a genetic counselor can be important in educating the individual about the condition. Infertility affects about 15% of couples wishing to have a child.[66] Intrinsic fertility cannot be restored in men with persistent azoospermia associated with seminiferous tubule failure or in women with ovarian failure. The counselor needs to understand how disappointing and indeed devastating this may be to some couples. While ART may offer hope, the couple should be aware that this is not necessarily an easy path and that success cannot be guaranteed. The genetic counselor, who acts as a member of the team caring for the individual, often plays an important role in helping the family to cope with the diagnosis both practically and emotionally. Several genes are involved in normal male reproductive functioning, and knowledge of these is important for the development of technologies for diagnosis so that comprehensive counseling can be provided to such men. At the minimum, testing in idiopathic and nonidiopathic male infertility should include semen analysis, hormonal evaluation, karyotyping, Yq microdeletion screening and mitochondrial mutation screening. This will aid in determining the etiology of infertility and the prognosis and appropriate management of such couples.

Technique for Blood Culture, Karyotyping, Yq Microdeletion Screening and Methylation Specific PCR

Chromosome Preparation from Peripheral Blood

Tissue culture technique has become an integral part of chromosome research. All tissue used for chromosome preparation is grown in culture. Lymphocytes are mononuclear cells that are used for cytogenetic diagnosis. Peripheral blood lymphocytes and leukemic cells from bone marrow grow as free-floating cells without attaching to the surface. These cultures are called suspension/hover cultures. Cells such as fibroblasts, epithelial cells from skin and amniocytes attach to the substrate and grow in layers. These cells are anchorage-dependent and are called monolayer cultures.

Circulating white blood cells (WBC) from peripheral blood of patients do not divide under routine culture conditions. Heparinized blood samples must be subjected to external stimulating factors or mitogens to induce mitosis. Mitogens include phytohemagglutinin (PHA), concana-valin A, pokeweed mitogen, protein A, Epstein-Barr virus (EBV) and phorbol ester.

Methodology of Peripheral Blood Culture and Karyotyping

Peripheral Blood Collection

- 5 ml peripheral blood is collected by venipuncture using a heparinized syringe.
- Depending on amount of blood collected, macroculture or microculture techniques are used.

Macroculture

This involves use of a peripheral blood specimen of adequate volume (> 5 ml). The blood cultures are set up by introducing plasma lymphocyte suspension (PLS) into culture medium.

- 5 ml peripheral blood is collected by venipuncture using a heparinized syringe.
- Sample is allowed to stand for 30-60 minutes.
- After 30 minutes, RBC sediment to lower half of the tube and lymphocyte and plasma remain in upper end of tube. This PLS is used for culture initiation.

Requirements

RPMI-1640 (GIBCO, Invitrogen, Carlsbad, Calif.): Store at 2-5°C for a month.

PHA (M form) (GIBCO): PHA is a glycoprotein that alters cell permeability and induces mitosis of small lymphocytes by stimulating synthesis of interleukins (IL). Store at 4°C for a few weeks.

Procedure

- Add 5 ml RPMI-1640 and 0.2 ml PHA to each culture vial. Plant two cultures per case.
- Hold the syringe vertically and tap gently to allow the mixing of buffy coat of the lymphocytes to the supernatant (plasma) without disturbing the lower layer of RBCs.
- Add 20-25 drops of lymphocyte-rich plasma (PLS) to each culture.
- Mix the contents of each culture vial gently.
- Incubate the culture for 72 hours at 37°C.
- Harvest the cultures on 3rd day at 72nd hour from the time of initiation.

Microculture

Blood specimens from neonates or polycythemic cases are quiet small in volume and do not yield an adequate volume of plasma. Cultures from such specimens are initiated using whole blood. This technique is called microculture technique.

Solutions

Growth medium RPMI-1640, fetal bovine serum (FBS) (heat inactivated), PHA.

Procedure

- Prepare culture vials by placing 4 ml of RPMI-1640, 1 ml of FBS and 0.2 ml of PHA in each vial. Add 0.5 ml whole blood to each vial.
- Incubate the cultures for 72 hours at 37°C.
- Harvest culture on 3rd day (72 hours).

Harvesting and Slide Preparation

PHA-stimulated cultures generally are harvested at the 72nd hour, since they yield a good mitotic index and chromosome morphology (cultures also can be harvested at 48 or 96 hours, but the metaphase index is low). Harvesting involves the removal of the colcemid followed by hypotonic treatment for 35-40 minutes and then fixation. Overnight fixation at 4°C strengthens the cell membrane and improves chromosome morphology. Fixation also is an important preliminary step for G-banding. The most popular stain for cytogenetic analysis is Giemsa (Romanavsky dye). Conventional staining using 4% Giemsa is very useful in studying satellites, dicentric chromosomes, ring chromosomes, fragments, double minutes, fragile sites, breaks and gaps.

Solutions

- Colcemid solution (10 µg/ml)(GIBCO):
  - Colcemid powder (5 mg) and distilled water.
- Hypotonic solution (0.075M KCl)
  - 56 mg KCl in 100 ml distilled water.
- Carnoy's fixative (3:1 methanol: acetic acid):
- 4% Giemsa stain (GIBCO).

Harvesting, Fixation of Chromosome Preparation and Staining

- Two hours prior to harvesting the cultures (70th hour) add 0.1 ml of colcemid (10 µg/ml) to each culture, mix by gently shaking and incubate the cultures for an additional 2 hours.
- Add the contents of each vial to 15 ml centrifuge tubes and centrifuge at 1000 rpm for 10 minutes.
- Discard the supernatant. Suspend the cell button in 5 ml of prewarmed hypotonic solution and incubate for 40-45 minutes at 37°C.
- After the hypotonic treatment, discard the supernatant and gently add 5 ml of freshly prepared, chilled Carnoy's fixative and mix well. Cover the tubes and store them overnight at 4°C.

- Next day again centrifuge the tubes at 1000 rpm for 10 minutes and suspend the cells in fresh fixative. Repeat these steps until pellet becomes white/pale yellow.
- After final centrifugation, when the cell pellet is absolutely white/pale yellow, suspend the cells in a small amount of fixative.
- Drop 2-3 drops of this suspension from a height of 0.3-1 meter on to chilled, clean slides that have a thin film of water.
- Stain the slides with Giemsa (4%).

G-banding and Karyotyping

G-banding is the benchmark for routine analysis of human chromosomes, producing a characteristic dark and light banding pattern. Chromatin structure and function vary along the length of the chromosome. Different staining techniques reflect the packaging, base composition and timing of DNA replication. Giemsa bands (G-bands) obtained by proteolytic digestion of the chromosomes with trypsin are the most widely used system in clinical laboratories for routine chromosome analysis. This technique is described as GTG-banding (G-bands by trypsin using Giemsa).

Solutions

- Trypsin solution (0.25%):
  - Trypsin (0.250) (GIBCO) 25 mg and 100 ml distilled water.
- Giemsa staining solution (2%):
  - Giemsa stain (GIBCO) 2 ml and 48 ml distilled water.

Procedure

- Age the air-dried slides for 3-4 days at room temperature, or overnight at 55-60°C.
- Treat the slides in trypsin solution (0.05%) for 15-20 seconds water.
- Rinse the slides briefly in normal saline.
- Stain the slides in 2% Giemsa's staining solution for 5 minutes.
- Rinse in distilled water and allow to air dry.
- Slides are examined under 100 × oil immersion.

Metaphase Screening

The G-banded slides are scanned for metaphase spreads under 10 × using a Carl Zeiss photomicroscope. The position of well-spread metaphases is recorded using the Vernier scale on the microscope stage. The metaphases are analyzed in detail under 100 × oil immersion objective. A minimum of 20-30 banded prometaphases and metaphases are captured using an image analyzer through a camera with a charge-coupled device (CCD). The metaphases are karyotyped using CytoVision software (Genetix, San Jose, California).

Photography and Karyotyping

Well-spread banded prometaphases and metaphases photographed under 100 × oil immersion objective and the automatic exposure system of Carl Zeiss photographic equipment using Copex Agfa Pan black and white film (Agfa, Ridgefield Park, NJ) are exposed and developed in Kodak's developer at 20°C using standard methods. Metaphase spreads are printed on normal resin-coated photographic paper (Sterling, No. 3). Photography printing is done using an Agfa enlarger with a 50 mm lens. Prints are developed and fixed using a standard print developer and fixer. Individual chromosomes are cut from the photographic prints and karyotypes are prepared according to the ideograms of the International System for Cytogenetic Nomenclature (ISCN, 1995), and the karyotypes are analyzed. Automated karyotyping with CytoVision software also may be done, reducing the time needed for analysis.

Chromosomal Analysis and Classification

Each species has a chromosomal complement characteristic in number and form known as karyotype, and the banding pattern of each chromosome is species and chromosome specific. A karyotype is prepared for analysis from a photographic image in which the stained chromosomes are arranged approximately in decreasing order of length and position of the centromere. For ease of analysis, the banding pattern can be represented diagrammatically as an ideogram. The chromosomes can be stained by a technique that gives a fairly uniform intensity (unbanded or solid stained) or by a technique that gives differential staining along the length of the chromosomes (banded). About 400 bands may be visible in each metaphase preparation and 550 to 700 bands in prometaphase stage cells. Although the chromosomes at this stage are much longer, several overlaps are present and the centromere is not easily distinguished in high resolution banding (HRB).

The classification of human chromosomes is currently decided by the Standing Committee on Human Cytogenetic Nomenclature who regularly updates the system (most recently in 1995). Their report, the ISCN, is the definitive reference for all cytogenetic nomenclature.

Protocol and Primers for PCR based Yq Microdeletion Screening

PCR microdeletion screening is carried out according to the guidelines of the European Academy of Andrology.[17] Recently we have published the use of a simplified multiplex PCR protocol and a list of primers for use in microdeletion screening in India.[19] Two nonpolymorphic primers are used for each AZF loci to confirm the diagnostic accuracy. The primer sets for AZFa loci (sY84, sY86); for AZFb loci (sY127, sY134) and for AZFc (sY254, sY255) are given in Table 15.1. The primer used as control is sY14 for the sex-determining region (SRY) on the short arm of the Y chromosome.

Amplification Procedure

For a PCR reaction volume of 50 µl: 50 ng genomic DNA, 20 pico mole of forward and reverse primers, 10 mM dNTPs, 25 mM $MgCl_2$. The Taq polymerase (Biotools, Madrid, Spain), 0.5 U per tube and buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100) provided by the manufacturer. DNA of normal healthy fertile males is used as the positive control and a fertile female as a negative control. Water is used as a blank. Initial denaturation is carried out at 95°C for 7 minutes 33 seconds. Following this, amplification is performed for 35 cycles as follows:

- 95°C for 1 minute (denaturation)
- 56°C for 30 seconds (annealing)
- 72°C for 1 minute (extension)
- A final extension step at 72°C for 7 minutes.

Gel Electrophoresis

Load 10 µl PCR-amplified products with 3 µl of bromophenol blue dye and electrophoresis through 1.8% Agarose gel. With each gel a DNA marker puC19 Msp1 digest is loaded to compare the exact position of the amplified products. If a sequence tagged site (STS) or gene fails to amplify, it is repeated in single PCR at least three times in the presence of internal control (SRY) as well as positive control. The deletions with respect to the various STS markers should be reconfirmed by using temperature gradient (lower annealing temperature). An STS is considered absent only after at least three amplification failures in the presence of successful amplification of internal control (SRY).

Epigenetic Alteration (Methylation Specific PCR)

Methylation-specific PCR (MSP) is the most extensively used method to study the DNA methylation pattern. MSP requires very small quantities of DNA and is sensitive to 0.1% methylated alleles of a given CpG island locus. MSP can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of cloning or methylation-sensitive restriction

Table 15.1: Primers used for STS-PCR Yq microdeletion analysis

S. no.	*Region*	*STS*	*Primer*	*Product Size (bp)*
1.	SRY	SRY-F SRY-R	5′-GAA TAT TCC CGC TCT CCG GA-3′ 5′-GCT GGT GCT CCA TTC TTG AG-3′	472
2.	AZFa	sY84-F sY84-R	5′-AGA AGG GTC TGA AAG CAG GT-3′ 5′-GCC TAC TAC CTG GAG GCT TC-3′	326
3.	AZFa	sY86-F sY86-R	5′-GTG ACA CAC AGA CTA TGC TTC-3 5′-ACA CAC AGA GGG ACA ACC CT -3′	320
4.	AZFb	sY127-F sY127-R	5′-GGC TCA CAA ACG AAA AGA AA -3′ 5′-CTG CAG GCA GTA ATA AGG GA-3′	274
5.	AZFb	sY134-F sY134-R	5′-GTCTGC CTC ACC ATA AAA CG -3′ 5′-ACC ACT GCC AAA ACT TTC AA -3′	301
6.	AZFc	sY254-F sY254-R	5′-GGG TGT TAC CAG AAG GCA AA -3′ 5′-GAA CCG TAT CTA CCA AAG CAG C -3′	400
7.	AZFc	sY255-F sY255-R	5′-GTT ACA GGA TTC GGC GTG AT -3′ 5′-CTC GTC ATG TGC AGC CAC -3′	126

enzymes. DNA is modified with sodium bisulphite treatment which converts all unmethylated, but not methylated, cytosine to uracil. Then DNA is amplified with primers, specific for methylated versus unmethylated DNA regions. All strategies assume that bisulfite-induced conversion of unmethylated cytosines to uracil is complete, and this serves as the basis of all subsequent techniques.

Sperm DNA is decondensed and extracted by adding 20 µl of alkaline lysis buffer, 1 M KOH (Merck, Darmstadt, Germany) and 0.05 M dithio-threitol (Invitrogen, Carlsbad, California), followed by incubation at 80°C during 20 min. The reaction is stopped by adding 20 µl of neutralizing buffer, 0.9 M Tris–HCl (Sigma, Steinheim, Germany), 0.3 M KCl and 0.2 M HCl, pH 8.3 (Merck). Extracted DNA is then treated and modified with a sodium bisulphite procedure using the CpGenome DNA Modification Kit (Chemicon International, Temecula, California), according to manufacturer's instructions. Bisulphite converts unmethylated cytosines to uracil, whereas 5-methylcytosine (5-MeC) remain unaltered. Only sequences with 95% of non-CpG cytosines converted and without unconverted cytosines adjacent to CpGs are validated. Then, the modified DNA is amplified by PCR.[67]

REFERENCES

1. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 2nd ed. Cambridge: Cambridge University Press; 1987.
2. Rowe P, Comhaire F, Hargreave TB, Mellows HJ. WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple. 1st ed. Cambridge: Cambridge University Press; 1993;15.
3. Dada R, Gupta NP, Kucheria K. Yq microdeletions—azoospermia factor candidate genes and spermatogenic arrest. J Biomol Tech. 2004;15:176-83.
4. Dada R, Kumar R, Ahmed ME, Kumar R, Sharma RS, Gupta NP, Mitra A, Gupta SK, Kucheria K. Clinical Implications of expanding phenotype IJPD. 2007;3:14-23.
5. Dohle GR, Halley DJ, Van Hemel JO, van den Ouwel AM, Pieters MH, Weber RF, Govaerts LC. Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. Hum Reprod. 2002;17:13-6.
6. Van Assche E, Bonduelle M, Tournaye H, Joris H, Verheyen G, Devroey P, Van Steirteghem A, Liebaers I. Cytogenetics of infertile men. Hum Reprod. 1996;11 Suppl 4:1-24; discussion 25-6.
7. Kumar R, Bhat A, Bamezai RN, Shamsi MB, Gupta NP, Ammini AC, Aron M, Sharma RK, Dada R. Necessity of nuclear and mitochondrial genome analysis prior to assisted reproductive techniques/intracytoplasmic sperm injection. Indian J Biochem Biophys. 2007;44:437-42.
8. Mau-Holzmann UA. Somatic chromosomal abnormalities in infertile men and women. Cytogenet Genome Res. 2005;111:317-36.
9. Kumar R, Shamsi MB, Ghaznavi MI, Jena M, Dada R. Structural chromosomal anomalies and their association with reproduction failure. Obs Gynae Today. 2007;12:152-4.
10. Chandley AC, Hargreave TB, McBeath S, Mitchell AR, Speed RM. Ring XY bivalent: a new phenomenon at metaphase I of meiosis in man. J Med Genet. 1987;24:101-6.
11. De Braekeleer M, Dao TN. Cytogenetic studies in male infertility: a review. Hum Reprod. 1991;6:245-50.
12. Calogero AE, De Palma A, Grazioso C, Barone N, Burrello N, Palermo I, Gulisano A, Pafumi C, D'Agata R. High sperm aneuploidy rate in unselected infertile patients and its relationship with intracytoplasmic sperm injection outcome. Hum Reprod. 2001;16:1433-9.
13. Anton E, Blanco J, Egozcue J, Vidal F. Sperm FISH studies in seven male carriers of Robertsonian translocation t(13;14)(q10;q10). Hum Reprod. 2004;19:1345-51.
14. Egozcue J, Blanco J, Anton E, Egozcue S, Sarrate Z, Vidal F. Genetic analysis of sperm and implications of severe male infertility—a review. Placenta. 2003;24 Suppl B:S62-5.
15. Tiepolo L, Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Genet. 1976;34:119-24.
16. Foresta C, Ferlin A, Moro E, Marin P, Rossi A, Scandellari C. [Microdeletion of chromosome Y in male infertility: role of the DAZ gene]. Ann Ital Med Int. 2001;16:82-92.
17. Simoni M, Bakker E, Eurlings MC, Matthijs G, Moro E, Muller CR, Vogt PH. Laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions. Int J Androl. 1999;22:292-9.
18. Dada R, Kumar R, Shamsi MB, Kucheria K, Sharma RK, Gupta SK, Gupta NP. Higher frequency of Yq microdeletions in sperm DNA as compared to DNA isolated from blood. Asian J Androl. 2007;9:720-2.
19. Mitra A, Dada R, Kumar R, Gupta NP, Kucheria K, Gupta SK. Screening for Y chromosome microdeletions in infertile Indian males: utility of simplified multiplex PCR. Indian J Med Res. 2008;127:124-32.
20. Zimmermann S, Schwärzler A, Buth S, Engel W, Adham IM. Transcription of the Leydig insulin-like gene is mediated by steroidogenic factor-1. Mol Endocrinol. 1988;12:706-13.
21. Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W, Adham IM. Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. Mol Endocrinol. 1999;13:681-91.
22. Ferguson Smith MA, Goodfellow PN. SRY and primary sex reversal syndromes. In: Scriver CR, Beaudet AL, Sly WS, Valle D (Eds). The metabolic and molecular basis of inherited disease 7th edn. New York: Mc-Graw Hill; 1995.

23. Dada R, Ahmad ME, Talwar R, Kucheria K. Clinical and Genetic study in a XX(SRY) negative male. Intl J Med (Medicine On Line).
24. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, Sham MH, Koopman P, Tam PP, Cheah KS. SOX9 directly regulates the type II collagen gene. Nat Genet. 1997;16:174-8.
25. Huang B, Wang S, Ning Y, Lamb AN, Bartley J. Autosomal XX sex reversal caused by duplication of SOX9. Am J Med Genet. 1999;87:349-53.
26. Swain A, Narvaez V, Burgoyne P, Camerino G, Lovell-Badge R. Dax1 antagonizes SRY action in mammalian sex determination. Nature. 1998;391:761-7.
27. Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, Moser C, Walker AP, McCabe ER, et al. An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature. 1994;372:635-41.
28. Folgero T, Bertheussen K, Lindal S, Torbergsen T, Oian P. Mitochondrial disease and reduced sperm motility. Hum Reprod. 1993;8:1863-8.
29. Kao SH, Chao HT, Wei YH. Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. Mol Hum Reprod. 1998;4:657-66.
30. Meinhardt A, Wilhelm B, Seitz J. Expression of mitochondrial marker proteins during spermatogenesis. Hum Reprod Update. 1999;5:108-19.
31. Shamsi MB, Kumar R, Bhat A, Bamezai RNK, Kumar R, Gupta NP, Das TK, Dada R. Mitochondrial DNA mutation in etiopathogenesis of male infertility. Ind J Urol. 2008;24:150-4.
32. Mundy AJ, Ryder TA, Edmonds DK. Asthenozoospermia and the human sperm mid-piece. Hum Reprod. 1995;10:116-9.
33. Sun ZM, Ding CF, Yan ZZ, Bao YZ. Ultrastructure and function of mitochondria in idiopathic asthenospermia: study of 151 cases. Zhonghua Yi Xue Za Zhi. 2007;87:1263-5.
34. Venkatesh S, Deecaraman M, Kumar R, Shamsi MB, Dada R. Reactive oxygen species and its role in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. Indian J Med Res. 2009;129:127-37.
35. Miller C, Saada A, Shaul N, Shabtai N, Ben-Shalom E, Shaag A, Hershkovitz E, Elpeleg O. Defective mitochondrial translation caused by a ribosomal protein (MRPS16) mutation. Ann Neurol. 2004;56:734-8.
36. Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev. 1998;78:547-81.
37. Wallace DC, Brown MD, Lott MT. Mitochondrial genetics. In: Rimoin DL, Connor JM, Pyeritz RE, Emery AEH editors. Emory and Rimoin's Principles and practice of medical genetics. London: Churchill Livingstone; 1997. p. 277-332.
38. Dada R, Kumar R, Shamsi MB, Tanwar M, Pathak D, Venkatesh S, Kumar M, Singh H, Singh K, Aron M, Kumar R, Singh G, Sharma RK, Gupta NP. Genetic screening in couples experiencing recurrent assisted procreation failure. Indian J Biochem Biophys. 2008; 45:611-5.
39. Barlow DP. Gametic imprinting in mammals. Science.1995;270:1610-3.
40. Murphy SK, Wylie AA, Jirtle RL. Imprinting of PEG3, the human homolog of a mouse gene involved in nurturing behavior. Genomics. 2001;71:110-7.
41. Reik W, Walter J. Genomic imprinting: parental influence on the genome. Nat Rev Genet. 2001;2:21-32.
42. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell. 1984;37:179-83.
43. Reik W, Collick A, Norris ML, Barton SC, Surani MA. Genomic imprinting determines methylation of parental alleles in transgenic mice. Nature. 1987;328:248-51.
44. Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. Nature. 1993;366:362-5.
45. Vu TH, Chuyen NV, Li T, Hoffman AR. Loss of imprinting of IGF2 sense and antisense transcripts in Wilms' tumor. Cancer Res. 2003;63:1900-5.
46. Henry I, Bonaiti-Pellie C, Chehensse V, Beldjord C, Schwartz C, Utermann G, Junien C. Uniparental paternal disomy in a genetic cancer-predisposing syndrome. Nature. 1991;351:665-7.
47. Weksberg R, Shen DR, Fei YL, Song QL, Squire J. Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. Nat Genet. 1993;5:143-50.
48. Kriaucionis S, Bird A. DNA methylation and Rett syndrome. Hum Mol Genet. 2003;12 Spec No 2:R221-7.
49. Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S. Establishment of male-specific epigenetic information. Gene. 2005;345:139-53.
50. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet. 2002;3:662-73.
51. Ariel M, Cedar H, McCarrey J. Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. Nat Genet. 1994;7:59-63.
52. Trasler JM. Origin and roles of genomic methylation patterns in male germ cells. Semin Cell Dev Biol. 1998;9:467-74.
53. de Lange T. Protection of mammalian telomeres. Oncogene. 2002;21:532-40.
54. Dionne I, Wellinger RJ. Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. Proc Natl Acad Sci USA. 1996;93:13902-7.
55. Hemann MT, Greider CW. G-strand overhangs on telomeres in telomerase-deficient mouse cells. Nucleic Acids Res. 1999;27:3964-9.
56. Watson JD. Origin of concatemeric T7 DNA. Nat New Biol. 1972;239:197-201.
57. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985;43:405-13.

58. Schrader M, Muller M, Heicappell R, Krause H, Schulze W, Miller K. Telomerase activity and expression of telomerase subunits in the testicular tissue of infertile patients. Fertil Steril. 2000;73:706-11.
59. Herrera E, Samper E, Martin-Caballero J, Flores JM, Lee HW, Blasco MA. Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J. 1999;18:2950-60.
60. Lenhard Rudolph K ed. Telomeres and Telomerase in Aging, Disease, and Cancer. Molecular Mechanisms of Adult Stem Cell Aging. Springer-Verlag, Berlin, Heidelberg; 2008.
61. Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S, Yamakido M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol. 1995;155:3711-5.
62. Harley CB, Futcher AB, Greider CW. Telomeres shorten during aging of human fibroblasts. Nature. 1990;345:458-60.
63. Cooke HJ, Smith BA. Variability at the telomeres of the human X/Y pseudoautosomal region. Cold Spring Harb Symp Quant Biol 1986;51:213-9.
64. Reiss MJ, Straughan R eds. Improving Nature. The Science and Ethics of Genetic Engineering Cambridge, UK.: Cambridge University Press; 1996.
65. Bennett RL, Hart KA, O'Rourke E, Barranger JA, Johnson J, MacDermot KD, Pastores GM, Steiner RD, Thadhani R. Fabry disease in genetic counseling practice: recommendations of the National Society of Genetic Counselors. J Genet Couns. 2002;11:121-46.
66. Foresta C, Ferlin A, Gianaroli L, Dallapiccola B. Guidelines for the appropriate use of genetic tests in infertile couples. Eur J Hum Genet. 2002;10:303-12.
67. Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, Sousa M. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. Mol Hum Reprod. 2008;14:67-74.

CHAPTER 16

Setting Up an Andrology Laboratory

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ABSTRACT

Male factor infertility is a sole or contributing factor in approximately 40% of the infertile couples and therefore investigating the cause of the male infertility constitutes a major part of the diagnosis and treatment of infertile couples. The andrology laboratory supports and conducts clinical assessment of the semen and helps in the diagnosis of various diseases and malfunctions related to male infertility.

Development of advanced assisted reproductive techniques in the last decade has increased the scope of today's andrology laboratories which are also involved in cryopreservation of gametes and reproductive tissues preserving the reproductive capacities of couples for various reasons.

This chapter serves as a practical guide for setting-up an andrology laboratory for diagnostic and therapeutic purposes.

INTRODUCTION

"Andrology" is the field of medicine that deals with matters affecting the male reproductive system. The earliest use of this term appeared in 1891 in the Journal of American Medical Association when it reported on the formation of the American Andrological Association.[1] Because male infertility is the primary or contributing factor in more than 40% of infertile couples [2], this field has been the object of constant research and has therefore seen advances in diagnostic tests and tools used for the investigation of male infertility.

The Andrology Laboratory encompasses clinical laboratories under both Pathology and Laboratory Medicine. It is entrusted with supporting clinical assessments of semen that may help diagnose potential diseases or malfunctions related to male fertility. Utilizing both simple and complex techniques, its evaluation provides useful information for the infertility specialist in the management of the subfertile man. Besides diagnostic activities, it should provide therapeutic procedures for patients requiring assistance in achieving pregnancy such as Assisted Reproduction techniques: Intrauterine Insemination (IUI) and In Vitro Fertilization (IVF). The Andrology laboratory also works in parallel with endocrinology, genetic, and in vitro fertilization laboratories to diagnose and assist in the treatment of patients facing difficulties conceiving or seeking preservation of their reproductive capacity by cryopreserving their gametes or reproductive tissue.

This chapter will provide readers, in a very practical way, with information on the basic requirements for setting up and operating an Andrology laboratory for diagnostic and therapeutic activities.

LABORATORY ASPECTS AND PROCEDURES

Numerous procedures can be performed in the Andrology Laboratory. Due to different applications (clinical and research) and clinical correlations in the current scientific literature, some of these procedures may not be included in this chapter.

Semen analysis is the most common test to be conducted in the Andrology Laboratory. It reveals how many spermatozoa are in the ejaculate, the vigor with which they move (quantitative motility), and how normal these cells look morphologically. Complementary assessments should include viability, the presence of

round cells and their identification (i.e. leukocytes or immature sperm cells), and detection of antibodies. Additional tests can be performed based on the results obtained from this initial analysis. Such tests may complement the initial analysis and provide more information about sperm cell functionality and structure. Biochemical markers such as Reactive Oxygen Species (ROS) and tests that assess sperm DNA damage and the inducibility of the Acrosome Reaction (AR) can provide a more specific functional assessment of these gametes. Bioassays that assess gamete interaction, such as the sperm-hamster egg penetration assay (SPA) and the human sperm-zona pellucida binding test (hemizona assay, HZA), evaluate the occurrence of spontaneous sperm AR and the capacity for penetration, respectively. They have strong diagnostic power but, like the AR test, still require better standardization before they can be introduced as clinical tools. [3] Moreover, the bioassays require hamster eggs and non-fertilizable human oocytes, which can impose extra practical limitations for their introduction into routine laboratory practice.

The most commonly performed tests are listed below. They can be classified in two groups: diagnostic and therapeutic.

Diagnostic Procedures

Complete Semen Analysis

Macroscopic parameters (color, volume, pH, viscosity, liquefaction, and agglutination)
Microscopic parameters (sperm concentration, motility, morphology, presence of round cells, and different types of debris)

Detection of Leukocytes in Semen

Myeloperoxidase test (Endtz)

Sperm Vitality and Membrane Viability

Eosin-Nigrosin test
Hypo-osmotic Swelling test (HOS)

Biochemical

Fructose qualitative and quantitative

Oxidative Stress

Reactive Oxygen Species (ROS)
Total Antioxidant Capacity (TAC)

Azoospermia Screen Procedure
Antibody Assessment

Test for anti-sperm antibodies on the sperm surface
Test for free antibodies, directed against the sperm surface (seminal plasma, serum or cervical mucus)

Sperm DNA Damage Test

Sperm Chromatin Structure Assay (SCSA)
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)
COMET, also called single cell gel electrophoresis (SCGE).

Therapeutic

Sperm Preparation for Intrauterine Insemination (IUI) or In vitro Fertilization (IVF)
Preparation of cryopreserved semen for IUI or IVF

Techniques

Gradient method
Swim-up method
Simple wash (concentration technique)

Cryopreservation

Semen
Epididymal aspirate
Testicular Tissue

LABORATORY DESIGN

Before working on any laboratory design, local regulations must be carefully studied. These regulations may differ between countries or even between states, and compliance is not optional.

Ideally, the Andrology Laboratory should contain distinct areas for diagnostic and therapeutic procedures (Figure 16.1). Therapeutic procedures should be performed in a sterile environment, which can be achieved by using a laminar flow hood. The physical area should be determined according to a pre-estimation of the number of tests to be offered by the service and the volume of procedures expected to be performed. This will determine the number of technicians that must be employed and the different types of equipment that must be made available such as microscopes, cell counters, incubators, etc. New laboratories may not have an established range of workload due to regional and market variations and thus should always keep in mind plans for future expansion.

Figure 16.1: Andrology Laboratroy and Reproductive Tissue Bank – Cleveland Clinic, Cleveland, Ohio

Even a small physical area can have its space optimized according to its disposition and organization. If the laboratory is going to offer cryopreservation, there should be space for the liquid nitrogen (lN_2) containers that will store tissue samples (Figure 16.2). Due to the chemical properties of lN_2, a laboratory should follow the proper safety guidelines for area ventilation and oxygen monitoring. Containers should be kept locked or in areas of restricted access. If containers are not checked daily, the laboratory should have alarms that sound when levels of lN_2 fall too low.

Semen Collection Room

The semen collection room (SCR) is a very important aspect of an Andrology Laboratory. Therefore, it must be planned with attention to design, especially in regards to location. Usually the SCR is located in proximity to the laboratory. Places that provide privacy (avoid busy corridors, lobbies or reception areas) are appropriate. The room can be equipped with a love seat or a small sofa and be supplied as needed with clean towels and sheets (Figure 16.3). There should be a sink or a washing station for use before and after collection. Not all collection rooms provide toilets and, if this is the case, a bathroom should be available nearby. The furnishings must be kept simple with preference for easy-to-clean materials or surfaces. Instructions for collection should be posted in the SCR even when they are explained to the patients by the nurse or laboratory technician. An interesting survey on patient satisfaction reported that the main areas of concern regarding the SCR were: sound privacy of the room, furnishing, cleanliness, and the availability and condition

Figure 16.2: Example of liquid nitrogen containers for storage

Figure 16.3: Example of semen collection room

of media.[4] The survey responders also rated videos as the most helpful media followed by magazines, with the preferred theme being heterosexual sex. In the same study, the responders preferred instructions on collection technique to be given face-to-face rather than in writing.

Office and Break Room

Clerical staff will also require office space. Patients' charts and files need to be kept within easy access of the laboratory staff. Computers and printers should be

available for entering results and work-related issues. A very important aspect is the break room. A resting area for employees must be included in the plan. Restrooms for employees should be close by.

Workstations

For practical purposes, workstations can be pre-defined. For example:

Workstation 1: Semen wet preparation (macroscopic parameters on seminal analysis, sperm count and motility, Endtz test, HOS and Eosin-Nigrosin preparations). This area can be equipped with microscopes, cell counters, incubators, water baths or warm stage and the necessary supplies and reagents (Figure 16.4). Controlled temperature at 37°C is needed for liquefaction, count and motility assessment, and HOS incubation. This workstation can also be equipped with an automated semen analyzer, which generates a more complete assessment of sperm motion characteristics. It is important to emphasize that the automated evaluation cannot replace a manual evaluation. It is still recommended that practitioners manually count semen samples to validate the computer results.

Workstation 2: Staining and slide preparation for morphology. This bench is usually near a sink or a drain where the slides can be washed during staining (Figure 16.5). Staining solutions can be kept in plastic containers or glassware and should be verified for possible contamination daily prior to use. When ready, slides are evaluated and second on a morphology assessment bench (Figure 16.6).

Figure 16.4: Example of Workstation 1

Figure 16.5: Example of Workstation 2

Figure 16.6: Example of morphology assessment bench

Workstation 3: Biochemical and antibody tests. Due to the nature of the fructose determination, a safety hood is recommended. Reagents for these procedures are usually kept refrigerated.

Workstation 4: Semen preparation and cryopreservation procedures. As mentioned previously, these procedures should be performed in a sterile environment (Figure 16.7). The same laminar flow hood, for example, could be used for both procedures; this could be an option if the lab cannot support two separate workstations logistically or financially. When optimizing the semen preparation procedures, a CO_2 incubator can be used for sample incubation in both the pre- and post-processing periods.

Figure 16.7: Example of Workstation 4

EQUIPMENT, SUPPLIES AND REAGENTS

The equipment, supplies, and reagents needed for each procedure vary according to the protocols to be followed. The following list suggests what each of the following tests may require:

Semen Analysis

Equipment

Phase Contrast Microscope with 10 × and 20 × Objective.
Cell Counter
Counting Chamber
Centrifuge (~1600 rpm)
Refrigerator (– 4 to – 8°Celsius)
Vortex mixer

Supplies

Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)
Automatic Pipetor (rechargeable)
Graduated Serological Pipets (1, 2 and 5 ml)
Test Tube rack for 15 mL tubes
15 mL Polystyrene Graduated Conical Centrifuge Tubes
Specimen Cups
Glass Microscope Slides and Coverslips
pH paper Range 6-8
Disposable Transfer Pipets (Pasteur Pipets)
Microcentrifuge tubes for dilution

Reagents

Tyrode's Salt Solution

Stain for Morphology

Equipment

Microscope with 100 × Objective
Differential cell Counter

Supplies

Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)
Glass Microscope Slides and Coverslips

Reagents

Cytoseal Mounting Media
Papanicolaou stain or Diff-Quik (rapid staining method) or Shorr stain

Endtz Test (Peroxidase Staining)

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Analytical Balance
Microscope 10 × objective

Supplies

Makler Chamber
Aluminum Foil
Dark Colored Microcentrifuge Tubes
Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)

Reagents

Ethanol, 96%
Benzidine
3% H_2O_2
Tyrodes Salt Solution

Eosin-Nigrosin Staining

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Microscope 100 × objective
Differential cell counter

Supplies

Disposable Transfer Pipets (Pasteur Pipets)
Boerner Slides (for mixing)
Glass Microscope Slides and Coverslips

Reagents

Stain components, Eosin Y
Cytoseal Mounting Media
Immersion Oil

Hypo-osmotic Swelling (HOS) Test

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Microscope 40 × Objective
Analytical Balance
Differential Cell Counter

Supplies

Microcentrifuge Tubes
Glass Microscope Slides and Coverslips
Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)

Reagents

Sodium Citrate
β-D Fructose
Distilled water

Fructose Qualitative

Equipment

Refrigerator (– 4 to – 8°Celsius)
Hot plate

Supplies

Pyrex test tube
Pyrex beaker

Reagents

Resorcinol
Concentrated HCl
Distilled water

Fructose Quantitative

Equipment

Refrigerator (– 4 to – 8°Celsius)
Spectrophotometer
77°C water bath

Supplies

100 mL beakers
50-mL Erlenmeyer flasks
Glass funnels
Filter paper
Serological pipet (5, 10 mL)
Micropipettes with corresponding tip (100 to 1000 µL)
13 × 100 glass test tubes with caps

Reagents

Concentrated HCl
D-Fructose (Levulose)
Deionized water
Resorcinol
Ethanol 95%

Sperm Antibody Testing by Immunobead Method

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Microscope 40 × Objective
Analytical Balance
Centrifuge (capable of 300 to 700 ×g)
56°C Water bath
Refrigerator (– 4 to – 8°Celsius)
pH meter
Differential Cell Counter
Covered Humidified Chamber

Supplies

15 mL Sterile Centrifuge Tubes with Caps
Micropipettes and Tips (8 µL and 200 µL)
5 mL Disposable Graduated Pipet
Glass Microscope Slides with Coverslips

Reagents

Bovine Serum Albumin
Tyrodes Salt Solution
Immunobead Rabbit Anti-Human Ig (H & L) Reagent
IgA Immunobeads
IgG Immunobeads
IgM Immunobeads

Reactive Oxygen Species

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Luminometer
Centrifuge

Supplies

15 mL Polystyrene Graduated Conical Tubes with caps
Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)
Graduated Serological Pipets (1, 2, and 5 mL)
Polystyrene round bottom tubes (6 mL)

Reagents

Dimethyl Sulfoxide (DMSO)
Luminol (5-amino-2,3 dehydro-1,4 phthalazinedione)
Phosphate Buffered Saline Solution 1X (PBS-1X)

Total Antioxidant Capacity

Equipment

Refrigerator (– 4 to – 8°Celsius)
Centrifuge
Plate shaker
Absorbance Microplate Reader
Micropipettes with different ranges and corresponding tips (20 µL, 100 µL, and 200 µL)

Supplies

Multichannel pipetor and tips (8 channels, 30 to 300 µL)
Aluminum foil
Microcentrifuge tubes
15-mL and 50-mL Polystyrene Graduated Conical Tubes
Round bottom tubes (12 x 75 mm)

Reagents

Antioxidant assay kit
Deionized water

Sperm DNA Damage per TUNEL Technique

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Flow cytometer

Supplies

Micropipettes with corresponding tips (200 µL, 1000 µL)
Counting chamber

Reagents

Commercial kit for detection or in-house preparation
3% paraformaldehyde
Microcentrifuge tubes
Ethanol

Gradient Sperm Wash

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

37°C Incubator (CO_2 supplied if possible)

Supplies

Sterile 15-mL Conical Centrifuge Tubes with Caps (tested against embryo and/or sperm toxicity)
Sterile Graduated Serological Pipets (1, 2, and 5 ml)
Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)
Sterile Disposable Transfer Pipets (Pasteur Pipets)
Sterile Specimen Containers (tested against embryo and/or sperm toxicity)

Reagents

Lower phase gradient
Upper phase gradient
Sperm Wash Media

Semen, Epididymal Aspiration, and Testicular Tissue Cryopreservation

* Equipment and supplies for semen analysis are required for pre-assessment

Equipment

Aliquot Mixer/Barnstead/Thermolyne
37 °C Incubator (CO_2 supplied if possible)
– 20 °C Freezer
Liquid nitrogen container
Liquid nitrogen container alarm
Oxygen monitor

Supplies

Sterile 15 mL Conical Centrifuge Tubes with Caps
Sterile Graduated Serological Pipets (1, 2, and 5 mL)
Micropipettes with different ranges and corresponding tips (5 µL, 10 µL, 20 µL, 100 µL)
Sterile Graduated Serological Pipets (1, 2, and 5 mL)
Sterile Specimen Containers (tested against embryo and/or sperm toxicity)
Test Tube rack for 15-mL tubes
Plastic Cryosleeves
Cryovials – 2 mL
Cryocanes
Laboratory Cryomarkers (non-toxic)
Liquid Nitrogen
Cryogenic Protective Gloves
Protective Eye Goggles

Reagents

Freezing Medium

Additional for Testicular Tissue

Equipment

Dissecting Microscope
Inverted Microscope

Supplies

Kontes Pellet Pestle
Sterile Tissue Culture Plates, 60 x 15 mm

Reagent

Sterile Mineral Oil

SAFETY GUIDELINES

The safety guidelines for the Andrology Laboratory include use of protective gloves and appropriate laboratory coats for all procedures. All samples should be treated as potentially contaminated and harmful. The World Health Organization (WHO) laboratory manual[5], in its fourth edition, has described these guidelines.

REFERENCES

1. Andrology The American Society of. A Handbook of Andrology. Lawrence: The American Society of Andrology; 1995.
2. Jeyendran RS. Protocols for Semen Analysis in Clinical Diagnosis. New York: The Parthenon Publishing Group; 2003.
3. Oehninger S, Franken D, Kruger T. Approaching the next millennium: How should we manage andrology diagnosis in the intracytoplasmic sperm injection era? Fertil Steril. 1997;67(3):434-6.
4. Levy BMCS, editor. The men's lounge improvement project: What do men really want? American Society for Reproductive Medicine; September, 2007. Elsevier Inc.
5. Kruger TF, Coetzee K. The role of sperm morphology in assisted reproduction. Hum Reprod Update. 1999;5(2):172-8.

CHAPTER 17

Quality Management of an Andrology Laboratory

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ABSTRACT

Andrology laboratories make use of various methods necessary for the assessment of human semen. Human semen assessments are designed to diagnose pathological conditions and malformations of the reproductive system. Many of the techniques used in the andrology laboratories are simple to follow but lack of standardization of these methods makes it difficult to compare results from different laboratories. This fact also affects the reliability and dependability of the study results.

The quality management programs consisting of quality control, quality assessment and quality improvement can be a solution for the standardization of these methods.

This chapter explains the importance of the quality management program in the andrology laboratories and helps in the development of a quality management plan for the laboratory.

INTRODUCTION

Theoretically, routine semen analysis is simple to perform place a drop of semen on a slide and determine the relative number, size/shape, and mobility of the spermatozoa. In practice, however, careful analysis of sperm concentration, movement, and morphology requires a great deal of technical expertise, procedural care, and meticulous quality control. Andrology procedures suffer from a lack of standardization, which has made semen analysis inaccurate and unreliable. As such, results can vary widely within and between laboratories. This has raised an urgent need for quality management (QM)—the integration of quality activities which include quality control (QC), quality assurance (QA), and quality improvement (QI)—into a management philosophy.[1]

This chapter will provide readers with an overview of QM and some basic tools that will help in the development of a QM plan in an andrology laboratory.

ANDROLOGY LABORATORY QM

The concept of QM, when applied to the andrology laboratory, can be described as a systematic program that monitors and evaluates the quality of the services that are being provided to patients. This includes identifying problems and finding their solutions as well as ensuring the quality of the laboratory services. Quality control in an andrology laboratory with standard operating procedures (SOP) should begin before any sample is collected and end with the presentation of results and communication to the clinician.

QA activities help assure that a product or service will satisfy its required quality characteristics. QC is the establishment of quality specifications for each piece of equipment or procedure and involves ensuring that they conform with established limits and standards. QI focuses on the progressive increase in the quality and efficiency of each aspect of the work and activities related to patient care and internal production.

Only a few publications on QM are available. De Jonge[2] numbered the steps that are involved in implementing a total quality management (TQM) program (market-in system): (1) define the service, (2) adopt and apply the principles of TQM, (3) develop mission and vision statements, (4) employ methods and tolls for continuous QI (*Plan-Do-Check-Act* cycle) and (5) continually enhance quality through creativity and innovation. In the *Plan-Do-Check-Act* cycle, the first step

is planning, which is followed by implementation. Results from the implementation are checked, and action is taken to improve the process. This cycle facilitates QI, as it is a never-ending and dynamic activity.

In 2002, the European Society of Human Reproduction and Embryology (ESHRE) [3] initiated a standardized course on Basic Semen Analysis and launched a Program for External Quality Assessment (EQA). The World Health Organization (WHO) Manual, in its fourth edition,[4] discusses technical QC issues for human semen evaluation. Mortimer, in 1994, described basic concepts of QC and QA.[5]

The QM program covers all areas of the laboratory. It must include a review of errors, complaints, number of incidents, patient satisfaction (surveys), and employee complaints and suggestions during a certain period predetermined by the lab director (at least annually). Pre-analytic, analytic and post-analytic variables must be evaluated. Pre-analytic processes include any step involved in the process prior to the testing (e.g. physicians' orders, patient instructions for semen collection, patient identification, sample transport, specimen acceptance criteria, and specimen identification). Post-analytic variables include any steps involved in the completion of the analytic phase (examination of the specimen) and results (e.g. turnaround time, quality and interpretability of reports).

A practical way to start a QM program in an Andrology Laboratory is to establish and monitor key indicators of quality. These indicators reflect activities critical to patient outcomes. The College of American Pathologists[6] suggests some key indicators:

- Patient/specimen identification
- Test order accuracy
- Start test turnaround time
- Critical value reporting

Documentation—System Control

Within a QM program, the laboratory must keep and control documentation involving numerous aspects that include but are not limited to the following:

- Organizational chart describing the relationship among the laboratory director, supervisor, clinical consultant and all other personnel
- List of current equipment and location
- Updated floor plan
- Current policies and procedures (annually reviewed by the director)
- Documented employee reviews of current policies and procedures related to their job activities
- Documentation of ongoing personnel meetings (employees and director) (e.g. agenda and meeting minutes)
- Evidence of a continuing clinical laboratory education program
- Updated personnel files of current employees
- Maintenance of discontinued procedures and policies for a determined period of time after date of retirement (local regulations may differ about the time of documents quarantine)
- Maintenance of patient records and materials related to test results for an appropriate length of time (local regulations may differ about the period)
- Procedures and policies that contain dates of implementation or review and identification of the reviewers
- The presence of an SOP manual that is easily accessible for employees (nearby work bench)
- Laboratory computer services system control (system functionality and reliability)
- Patient data (automated or manual: documentation, process and review of reports)
- Inventory and storage of supplies (effective control system, e.g. quantity, storage temperature monitoring)
- Safety policies that are periodically reviewed and readily available for all employees (fire drills and fire extinguisher training may be required)
- A chemical hygiene plan (CHP) that defines the safety procedures for hazardous chemicals used in the laboratory
- A material safety data sheets (MSDS), which should be readily available and current
- Policy for emergency power supply (to ensure preservation of patients' specimens, e.g. refrigerators, freezers and incubators).

Proficiency Testing

Some laboratories may be required to participate in a proficiency testing (PT). PT can be defined as *"determination of laboratory testing performance by means of inter-laboratory comparisons, in which a PT program periodically sends multiple specimens to members of a group of laboratories for analysis; the program then compares each laboratory's results with those of other laboratories in the group and/or with an assigned value"*.[6] Interlaboratory communication is prohibited, and the PT samples should

be integrated within the routine workload, which means the PT sample should be treated as a patient sample. Personnel who routinely test patient samples will be required to analyze the PT samples using the same protocol. These services should be performed in accordance with written procedures.

Laboratory Accreditation

The laboratory must have policies that ensure it is in compliance with applicable state and local laws and regulations. Besides being in compliance with regulations and having the required licenses to operate, some laboratories are required to be accredited by non-government organizations. In the accreditation process, the lab will conduct self inspections and receive peer-assessment by the selected authority. During these inspections, the lab will be evaluated for compliance with pre-established standards (specification and criteria) for practices, procedures, equipment (e.g. calibration ranges maintenance), and reagents (e.g. expiration dates, storage) involved in the procedures offered by the laboratory. The accreditation process may include evaluation of the facility QM program, proficiency testing, pre-analytic, analytic and post-analytic variables for sample testing, test method validation, laboratory computer services, personnel, physical facilities, and laboratory safety. Effective accreditation schemes share the same basic characteristics (Figure 17.1).

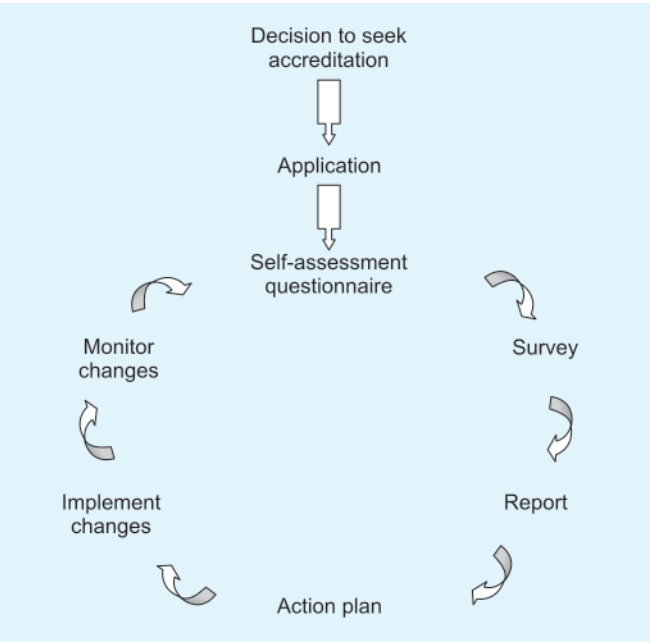

Figure 17.1: Generic accreditation process (Adapted from Mortimer and Mortimer, 2005[1])

ISO Standards

According to Mortimer and Mortimer[1], the International Organization for Standardization or ISO based in Geneva develops standards according to the essential principles of: consensus, industry-wide solutions and voluntary involvement. The ISO 15189:2003 *Medical Laboratories—Particular requirements for Quality Competence* is the standard most relevant to Andrology and in vitro fertilization (IVF) labs, and laboratory activities must comply with this ISO. From the same source one can find information about the European Tissues and Cells Directive that was passed on March, 31st of 2004-*Directive* 2004/23/EC. *On setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells* – which specifically includes reproductive cells and stem cells for human transplantation. Article 6 of this directive requires that all "tissue establishments," where any of the above mentioned activities are undertaken, are to be accredited, designated, authorized, or licensed by a competent authority of the Member States for the purpose of those activities, with legal compliance required no later than April, 7th, 2006. Therefore, any laboratory in Europe that processes semen for Intrauterine Insemination (IUI) is subjected to this regulation.

Quality Control

QC in the andrology laboratory has been an issue for many years. Many studies have discussed the lack of operational standards, discrepancy, imprecision, and inconsistency involved in semen analysis.[7-9]

The accurate assessment of conventional semen tests remains problematic even for the experienced technologist. Considerable inter- and intra-laboratory variability exists in regards to these assessments, partly because a wide variety of techniques are used by different laboratories. Secondly, the experience level of the technical personnel performing these assessments varies. The use of uniform standardized laboratory techniques aligned with the maintenance of a comprehensive quality control program is warranted to reduce this variability in andrology services.

One of the first steps for introducing a QC system in an andrology laboratory is to have an actual written QC program. It should start with a complete SOP manual that will be followed by each and every employee engaged in patient testing.

Well-trained employees are essential for the QA of an andrology laboratory. Although descriptions of QC systems tend to focus almost exclusively on the training of technicians to assure the quality of sample analysis, operator error is only one component of the total analytical error in any method.[7] A well-trained technician can not produce precise and accurate results using non-standardized, subjective, and non-repeatable methods. Therefore, training each technician in a standardized manner (based on well elaborated, objective and repeatable SOPs) will help establish uniformity in the laboratory evaluation. Competency checks with feedback and re-training in an ongoing manner is essential for an accurate assessment and reproducibility of the techniques. A control system for results should be implemented that includes recording test data, identifying each person responsible for the evaluation, and trouble-shooting when required.

Practical Guide

The SOPs play an important role for any QC program. Their elaboration should follow pre-established formats and standards published by local or international institutes such as the "Clinical and Laboratory Standards Institute, CLSI, NCCLS" or any other governing national requirements. An efficient SOP needs to offer all the necessary information for the test to be reproducible and to be performed efficiently. It could be, for an example, schematized with the following topics:

SOP-model

- Name of the test
- Introduction
- Principle of procedure (goals and more general information about the test)
- Specimen collection (patient's instruction, transport, identification)
- Equipment and materials
- Reagents
- Quality control (negative and positive control if required)
- Procedure description (detailed, numbering and explaining step by step)
- Calculations (if required)
- Normal ranges (reference values for normality)
- Panic values (if applicable)
- Assay performance (inter and intra-assay, variability of the control, sensitivity)
- References (articles published, current literature, in-house elaboration).

The following check list can be used to implement or complement an andrology laboratory QC program. Each piece of equipment should contain a binder or file that contains the QC sheets as well as records of maintenance and possible occurrences (e.g. malfunctioning or breakage). Each check should have the initials of the person performing the QC and the date.

QC Checklist

- **Daily Check-QC tasks**
  - Temperatures (room, refrigerators, freezers, incubators)
  - Liquid nitrogen tanks (levels and order refill)
  - Microscopes (optics, cleaning, covering)
  - Clean bench tops with 10% Clorox solution
  - Morphology staining solutions (contamination, debris, change in color).
- **Weekly Check-QC tasks**
  - Incubators (change water in tray)
  - Liquid nitrogen tanks (Fill)
  - pH meter (level of KCl/AgCl in electrodes, calibration, change soak solution)
  - Automated semen analyzer (beads calibration, motility QC, review of gate settings)
  - Glassware washing (residual contamination)
  - Waterbaths (temperature and levels as used)
  - Endtz test working solution (contamination, debris, change in color)
  - Tyrodes buffer solution (contamination, debris, change in color)
  - Eye wash (testing water flow).
- **Monthly Check-QC tasks**
  - Incubator (clean interior and exterior)
  - Microscopes (mechanic system, illumination system, clean exterior and phase contrast)
  - Automated semen analyzer (clean interior and exterior)
  - Spectrophotometer (wavelength, clean interior and exterior, absorbance and linearity)
  - Eosin-Nigrosin staining solutions (contamination, debris, change in color)
  - HOS solution (contamination, debris, change in color).

- **Quarterly Check-QC tasks**
  - Refrigerators/freezers (clean interior and exterior)
  - Centrifuges (clean interior and exterior).
- **Bi-annually Check-QC tasks**
  - Balances (S class weights)
  - Balances (blind spheres)
  - Micropipettes and pipettes (calibration)
  - Semi-annual environment cultures (workstations: semen wet preparation and therapeutic).
- **Annually Check-QC tasks**
  - Thermometers (calibration)
  - Timers (calibration)
  - All equipment ground and mechanical check
- **Check-As used**
  - Laminar flow/safety cabinet hood (clean before and after use).
- **Check-As received**

  Supplies and reagents
  - Receiving date
  - Product characteristics (temperature during transportation and during arrival)
  - Opening date
  - Expiration date
  - Storage requirements
  - Toxicity testing (for every new lot, e.g. sperm or mouse embryo survival).

Hands-on Training

Ideally, training should be offered during one-on-one practice sessions. For a basic training in seminal analysis, the following topics can be covered:

- Discussion about the subject and introduction of the techniques to be learned (recent literature, test objective, and parameters to be analyzed)
- Microscope alignment (setting up and focusing, setting stage, calibration and calculating count factor)
- Patient orientation (instructions for collection and sample transportation if applicable)
- Specimen collection and handling (discuss viscous semen samples)
- Macroscopic parameter evaluation
- Sperm count evaluation (including different dilutions)
- Sperm progression and motility evaluation
- Quality control on sperm count and motility (instructions, count forms, use of monitor grids)
- Calculations of coefficient of variation and repeated analysis when required (basic statistical analysis can be used, e.g. a 15% or less coefficient of error for intratech variability (CV), and 15% or less for the mean percentage difference from the standard could be required)
- Morphology slides preparation (semen smears: proper labeling, staining and storage of slides)
- Morphology differential scoring by determined criteria WHO [10] or Strict[11] as described in the SOP
- Calculations of coefficient of variation and repeated scoring when required
- Different samples from patients and healthy semen donors should be evaluated
- Troubleshooting (emphasis on sperm identification, proper mixing, loading, counting, motility and progression assessment, morphology scoring and quality control)
- Training update and individual feedback
- Laboratory worksheets, source documents, and data entry
- Competency check: proficiency test slides
- Review of safety guidelines and good laboratory practices (GLP).

Staffing Issues

The QM program should be a philosophy of work for a laboratory. Strong leadership is necessary before employees can embrace this philosophy. An organization is only as good as its leadership, because a good leader establishes unity of purpose, direction, and the internal environment in which employees can become fully involved in achieving the organization's objectives.[2] A healthy relationship among directors, supervisors and technicians in which the concept of "team work" is applied improves communication and productivity. Open communication and empowered employees are ingredients for success. Staff issues such as insufficient, poorly trained or inexperienced staff, overwork, and lack of guidance will directly interfere with the quality of any program. A combination of these factors can ruin any service.

REFERENCES

1. Mortimer M. Quality and Risk Management in the IVF Laboratory. Cambridge: Cambridge University Press; 2005.
2. De Jonge C. Commentary: forging a partnership between total quality management and the andrology laboratory. J Androl 2000;21(2):203-.5.
3. ESHRE. Manual on Basic Semen Analysis. European Society of Human Reproduction and Embryology 2002.

4. Kruger TF, Coetzee K. The role of sperm morphology in assisted reproduction. Hum Reprod Update. 1999;5(2):172-78.
5. Mortimer D. Practical Laboratory Andrology. Oxford, editor. New York: Oxford University Press 1994.
6. Francis Sharkey M, editor. Laboratory Accreditation Manual: College of American Pathologists 2007.
7. Clements S, Cooke ID, Barratt CL. Implementing comprehensive quality control in the andrology laboratory. Hum Reprod 1995;10(8):2096-106.
8. Macleod IC, Irvine DS, Masterton A, Taylor A, Templeton AA. Assessment of the conventional criteria of semen quality by computer-assisted image analysis: evaluation of the Hamilton-Thorn motility analyser in the context of a service andrology laboratory. Hum Reprod 1994;9(2):310-19.
9. Mortimer D, Shu MA, Tan R. Standardization and quality control of sperm concentration and sperm motility counts in semen analysis. Hum Reprod 1986;1(5):299-303.
10. World Health Organization: WHO Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge: Cambridge University Press; 1992 Contract No.: Document Number.
11. Kruger TF. Atlas of Human Sperm Morphology Evaluation. First ed. London: Taylor & Francis 2004.

SECTION 4

Human Spermatozoa in Assisted Conception

CHAPTER 18

Sperm Selection in Assisted Reproductive Techniques

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ABSTRACT

The application of assisted reproductive techniques (ART) has provided help to many men seeking to father a child, although the current success rates of these procedures remain suboptimal. Selection of human spermatozoa prior to ART currently is based on criteria that include viability, motility and morphology. The first sperm separation methods to be developed involved washing procedures with subsequent resuspension of the male germ cells. Double-density gradient centrifugation (DGC) and the swim-up procedure are currently standard preparation techniques. Glass wool filtration (GWF) is also known to provide sperm samples with comparable recovery rates, motility, morphology, and fertilizing capacity. Advanced protocols allow for selecting sperm according to their ultrastructural morphology or surface charges by electrophoresis.

New insights into the molecular biology of spermatozoa have prompted the development of molecular selection strategies, including hyaluronic acid-mediated sperm selection, annexin V magnetic- activated cell separation (MACS), and annexin V molecular glass wool filtration. Sperm preparation that combines MACS with double-density centrifugation provides spermatozoa of higher quality in terms of motility, viability and apoptosis indices compared with other conventional sperm preparation methods. Furthermore, sperm prepared according to this protocol showed improved ability to fertilize eggs using the hamster oocyte penetration assay.

INTRODUCTION

The application of assisted reproductive techniques (ART) has provided help to many men seeking to father a child, although the current success rates of these procedures remain suboptimal (Society for Assisted Reproductive Technology and American Society for Reproductive Medicine, 2004). Since raw semen cannot be used in most assisted reproductive techniques, a workup of the ejaculate is needed to extract those sperm that are capable to fertilize the egg.

The first sperm separation methods developed embraced one- or two-step washing procedures with subsequent resuspension of the male germ cells (Edwards et al, 1969). Double-density gradient centrifugation (DGC) and the swim-up procedure are currently standard preparation techniques. Glass wool filtration (GWF) is also known to provide sperm samples with comparable recovery rates, motility, morphology, and fertilizing capacity (Henkel and Schill, 2003; Holmgren and Jeyendran, 1993; Johnson et al, 1996; Van Den et al, 1997a).

Advanced protocols that allow sperm to be selected according to their ultrastructural morphology (Bartoov et al, 2002) or surface charges by electrophoresis (Ainsworth et al, 2005) overcome the limitations of these classical separation procedures. New insights into the molecular biology of spermatozoa have prompted the development of molecular selection strategies, including hyaluronic acid-mediated sperm selection (Huszar et al, 2006), annexin V magnetic-activated cell separation (MACS), and annexin V molecular glass wool filtration (Grunewald et al, 2001; Paasch et al, 2003b).

CONVENTIONAL SELECTION STRATEGIES

Optimization of assisted conception outcomes involves the development of rapid, safe are effective techniques for the isolation of functional human spermatozoa that are free from significant DNA damage. Over the last decades a variety of standard procedures have been

developed with certain modifications (conventional selection strategies). These sperm selection techniques can be classified by their basis on centrifugation, filtration or sperm migration. Among the centrifugation techniques, density gradient centrifugation (DGC) has been proposed as the gold standard for sperm preparation. The latest developments in sperm selection are focused on the sperm surface with or without a standard preparation protocol (molecular selection strategies).

CENTRIFUGATION TECHNIQUES

Simple Sperm Wash

The one-step washing technique is considered a good alternative for processing certain compromised samples (Srisombut et al, 1998). Simple centrifugation is known to cause definite harm to the cells and therefore should not be applied for routine sperm processing.

Density Gradient Centrifugation (DGC)

The semen sample is placed on top of a density gradient and centrifuged for 15–30 minutes. During this procedure, highly motile spermatozoa move actively in the direction of the sedimentation gradient and therefore can reach lower areas quicker than poorly motile or immotile cells. Finally, highly purified motile sperm cells are enriched in the soft pellet at the bottom.

This method allows for the enrichment of motile sperm (multiplicative factor of 1.2 to 2.1) and morphologically normal sperm (multiplicative factor of 1.2 to 1.8) (Glander, 1993). In principal, continuous or discontinuous gradients are in use for DGC (Bolton and Braude, 1984; Pousette et al, 1986). The former comprises a gradually increasing density towards the bottom of the vial, whereas the latter is characterized by clear boundaries between the different densities of the gradient.

A recent study demonstrated that DGC with two wash steps results in a higher sperm motility and a better pregnancy rate after intrauterine insemination in comparison to a single wash after DGC (Öztürk Turhan et al, 2008). Various separation media for DGC have been introduced over time, such as Ficoll® (Chalfont St. Giles, UK) (Bongso et al, 1989; Harrison, 1976), dextran-visotrast (Glander et al, 1990), Nycodenz (Gentaur, Brussels, Belgium) (Gellert-Mortimer et al, 1988) and Percoll®, polyvinylpyrro-lidone (PVP)-coated silica particles (GE Healthcare Life Sciences, Uppsala, Sweden) (Gorus and Pipeleers, 1981). Percoll® – DGC has been demonstrated to be beneficial for all methods of assisted reproduction (Mortimer and Mortimer, 1992). Comparison of the discontinuous Percoll® gradient with other techniques for separation of motile sperm indicated that the discontinuous Percoll® gradient has advantages in terms of recovery, enhancement of motility, and increased ability to penetrate zona-free hamster ova (Berger et al, 1985).

Because of the risk of contamination with endotoxins, possible membrane alterations and inflammatory responses that could be induced by the insemination of sperm populations contaminated with Percoll®, it was withdrawn from the market for clinical use in assisted reproduction in October 1996 (Pharmacia Biotech, 1996; Pickering et al, 1989) and replacement products such as IxaPrep® (Medicult, Copenhagen, Denmark), PureSperm® (Sepal Reproductive Devices, Boston, Mass.), SilSelect (FertiPro NV, Beernem, Belgium)® and Isolate® (Irvine Scientific, Santa Ana, Calif.) have been introduced (Makkar et al, 1999; Sills et al, 2002). All these less toxic media contain silane-coated silica particles. The quality of sperm prepared with these media seems to be equivalent compared with Percoll® in regard to recovery rate, motility, viability, normal sperm morphology and velocity (Chen and Bongso, 1999; Claassens et al, 1998; Soderlund and Lundin, 2000) although there are conflicting reports for motility (McCann and Chantler, 2000).

Nitric oxide (NO) production has been found to be lower using IxaPrep® as a replacement for Percoll. NO adversely affects sperm motility, sperm-zona binding and embryonic development, suggesting that using these newer replacement media offer an overall advantage in ART (Wu et al, 2004). Several studies have demonstrated that samples processed by DGC displayed fewer indicators of apoptotic somatic cells such as caspase activation, disruption of mitochondrial membrane potential (MMP), DNA-fragmentation, and externalized phosphatidylserine (PS) (Barroso et al, 2006; Larson et al, 1999a; Ollero et al, 2001; Paasch et al, 2003a).

To further improve the quality of sperm, a combination of washing procedures or DGC and swim-up is widely used (Scott, Jr. et al, 1989). The combined use of DGC and a revised swim-up was demonstrated to remove HIV-1 RNA (Savasi et al, 2008) and pro-viral DNA (Kato et al, 2006). Single sperm washing also has been shown to

achieve complete virus decontamination. These findings suggest that even semen from HIV-positive men with oligoazoospermia can be used for intracytoplasmic sperm injection (ICSI) (Bostan et al, 2008).

FILTRATION TECHNIQUES

Glass Wool Filtration (GWF)

The principle of glass wool filtration for sperm preparation was introduced 30 years ago. (Paulson et al, 1979; Paulson and Polakoski, 1977). This technique offers the advantage of processing the whole ejaculate, followed by an additional centrifugation step to remove the seminal plasma. Highly motile spermatozoa are separated from immotile sperm cells by means of densely packed glass wool fibers using gravitational forces and the self-propelled motion of the cells. The efficacy of each filtration run is directly dependent on the properties of the glass wool used (Sanchez et al, 1996). The chemical nature of the glass (i.e. borate, silicate or quartz), its surface structure, charge and thickness of the glass wool fibers directly influence filtration efficacy (Ford et al, 1992). Potential risks of the technique are damages to the membrane and acrosome or the transmission of glass particles into the filtrate (Sherman et al, 1981). The glass wool (Code # 112, Manville Fiber Glass Corp., Denver, Colo.), available as SpermFertil® columns (TransMIT GmbH, Giessen, Germany) has been tested extensively (Grunewald et al, 2007).

Glasswool filtration separates human spermatozoa according to motility and size of the sperm head. Sperm head size is closely correlated with the chromatin condensation quality and DNA fragmentation as measured by the sperm chromatin structure assay (SCSA) (Henkel et al, 1994b; Larson et al, 1999b). Reactive oxygen species (ROS) are known to induce DNA fragmentation in sperm, which in turn are directly and negatively correlated to the outcome of ART (Greco et al, 2005). GWF eliminates up to 90% of leukocytes (Sanchez et al, 1996), leading to a significant reduction of ROS in the sample, thereby contributing to the prevention of sperm DNA damage (Henkel and Schill, 1998). In comparison to Percoll-DGC, glass wool filtration yields more functionally intact spermatozoa of superior quality (Johnson et al, 1996), without affecting fertilization rate and embryo quality in an ICSI program (Van Den et al, 1997b). However these positive effects are partly related in part to initial sperm concentration (Rhemrev et al, 1989). Compared with swim-up techniques, GWF yields a higher recovery of viable and potentially fertile spermatozoa (Coetzee et al, 1994; Holmgren and Jeyendran, 1993; Van der Ven et al, 1988). On the molecular level, samples processed by GWF are shown to be low in caspase activation, MMP disruption, DNA fragmentation and externalized phosphatidylserine (PS) (Larson et al, 1999a; Rasch et al, 2005).

Glass Bead Filtration (GBF)

The ability of selected glass surfaces to sort out leukocytes and sperm with damaged membranes led to the concept of using these features in a system that provides a relatively smart surface instead of the unpredictable glass wool surface. Spheres made of glass arranged in a column meet these criteria (Daya et al, 1987). Glass bead filtration systems filter spermatozoa that have significantly better progressive motility and viability and enhanced fertilizing capacity (Daya and Gwatkin, 1987). Samples processed with GBF provided higher counts of motile sperm than with swim-up preparation (Lechtzin et al, 1991), although with less velocity and linearity (Calamera et al, 1991). GBF before cryopreservation significantly enhanced post-thaw sperm quality (Klinc et al, 2005). Concerns regarding the possibility of transferring glass spheres into filtrate, however, prevented a wider acceptance of this technique.

Sephadex Columns

Sephadex bead sperm preparation was introduced into ART in the early 1990s (Drobnis et al, 1991). This gel filtration technique is based on a chromatographic media composed of macroscopic beads synthetically derived from the polysaccharide dextran. SpermPrep® (ZDL, Lexington, Ky.), is a commercial kit that has been intensively investigated. Compared to migration-sedimentation and swim-up, it provides significantly higher yields (Gabriel and Vawda, 1993) and more motile spermatozoa than obtained by DGC (Ohashi et al, 1992). In comparison to swim-up, morphologically normal sperm are enriched in the filtrate and significantly higher pregnancy rates for intrauterine insemination are reported (Zavos and Centola, 1992). However, spermatozoa showed a significantly higher percentage of normally chromatin-condensed and morphologically normal spermatozoa after DGC, although fertilization rates were similar (Hammadeh et al, 2001).

MIGRATION TECHNIQUES

The self-propelled movement of spermatozoa is an essential prerequisite for all migration methods and guarantees a very clean workup.

Swim-up Procedure

The swim-up procedure uses the active motion of spermatozoa. Intact moving cells swim out of a pellet derived by a simple washing step into an overlaid media for 30-60 minutes. Highly motile, morphologically intact spermatozoa are enriched in the absence of other cells, proteins and debris within the supernatant (Dominguez et al, 1999). Anti-gravitational centrifugation has been proposed to shorten preparation time (Babbo et al, 1999).

The benefit of this method may be limited due to the fact that close cell-to-cell contact of sperm with each other, debris and other substances may lead to extensive ROS production and consecutive DNA damage (Twigg et al, 1998), although this could not be verified (Younglai et al, 2001). Swim-up performed directly from the ejaculate without pre-wash and other technical modifications are attempts to reduce ROS production and to increase the total number of recovered spermatozoa, which may also be used for ICSI (Al Hasani et al, 1995). The use of a highly purified preparation of hyaluronic acid concentrated 1 mg/ml in culture medium (SpermSelect™, GE Healthcare Life Sciences) resulted in a significantly higher recovery of motile spermatozoa and higher pregnancy rates in a clinical IVF program (Wikland et al, 1987). When compared with gradient centrifugation, the clearing efficacy after swim-up is lower for disomies and higher for diploidies (Henkel et al, 1994a; Jakab et al, 2003). Swim-up prior to freezing improved sperm vitality in cryopreserved oligozoospermic samples to a level comparable to that achieved by DGC (Counsel et al, 2004). All things considered the swim-up procedure is a confident and effective method for the purpose of intrauterine insemination (IUI) (Jameel, 2008).

Migration-Sedimentation

This method combines a direct swim-up procedure with an additional sedimentation step requiring a special glass or plastic tube with an inner cone (Tea et al, 1983). Although modifications have been proposed and spermatozoa obtained are of good quality in terms of their motility pattern, the method has not been established widely in ART due to its low yield (Yavetz et al, 1996; Yener et al, 1990). However, as sperm characteristics correlated significantly with their fertilizing capacity, this method could be used in evaluation of sperm function (Hauser et al, 1992).

Transmembrane Migration

This sperm preparation technique uses filtration through a membrane filter with cylindrical pores at right angles to the plane of the membrane (Chijioke et al, 1988). Due to a low ratio of the total cross-sectional area of the pores to the overall membrane area, the yield is extremely low. L4 membranes selective for spermatozoa with normal membrane integrity have been introduced with a simultaneous increase in motility and significant depletion of leukocytes (Agarwal et al, 1992). Despite these improvements, the transmembrane migration technique has never come into practical clinical use for human assisted reproduction.

Advanced Sperm Selection Methods

Ultrastructural Sperm Selection

The ultrastructural morphology of the sperm head components have been correlated with sperm fertilizing capacity *in vitro* (Mashiach et al, 1992). The examination is performed in real time using an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification up to 6300×. The motile sperm organelle morphology examination (MSOME) method was significantly and positively associated with both fertilization rate and pregnancy outcome (Bartoov et al, 2002). ICSI performed with selected spermatozoa with strictly defined, morphologically normal nuclei significantly improves the incidence of pregnancy in couples with previous ICSI failures (Bartoov et al, 2003), particularly when the use of sperm with vacuoles is avoided. Recently a conductive correlation between an increase in DNA fragmentation and the presence of spermatozoa with large nuclear vacuoles (LNV) was shown. A correlation between a higher fraction of denatured DNA and LNV also was found. These results support the routine selection of spermatozoa by MSOME (Franco Jr et al, 2008).

Electrophoretic Sperm Isolation

A novel approach to sperm isolation based on electrophoretic sperm separation by size and charge has been described recently (Ainsworth et al, 2005).

Electrophoresis-based microflow technology for the separation of spermatozoa by size and charge consists of two outer chambers separated from two inner chambers by polyacrylamide restriction membranes with a pore size of 15 kDa. The pore size allows directional movement of competent spermatozoa in the applied electric field and the size-exclusion of contaminating cell populations. Large numbers of spermatozoa that are viable and morphologically normal can be selected. The resulting cell population shows a low incidence of DNA damage and contaminating cells and compared favorably with DGC in purity of the sperm population and lack of ROS generation, as well as the viability and morphological integrity of the isolated cells. However, electrophoresis of spermatozoa is detrimental to their motility (Ainsworth et al, 2005).

Molecular Selection Strategies

Hyaluronic Acid-Mediated Sperm Selection

Hyaluronic acid or hyaluronan, a linear repeating disaccharide polymer, increases the motility and viability of freshly ejaculated or cryopreserved-thawed human spermatozoa when added to sperm preparation media (Huszar et al, 1990).

Hyaluronic acid-mediated sperm selection is a novel technique that is comparable to sperm-zona pellucida binding. The presence of hyaluronic acid receptor on the plasma membrane of mature acrosome-intact sperm, coupled with hyaluronic acid-coated glass or plastic surfaces, facilitates selection of single mature sperm (Huszar et al, 2006). The frequencies of sperm with chromosomal disomy are reduced approximately four- to five-fold in hyaluronic acid-selected sperm compared with semen sperm compensating the increase in such abnormalities in intracytoplasmic sperm injection offspring. Hyaluronic acid binding also excludes immature sperm with cytoplasmic extrusion, persistent histones and DNA chain breaks (Huszar et al, 2003). Recent studies did not confirm the efficacy of hyaluronan-binding assay (HAB) in improving the selection of motile spermatozoa without DNA damage (Pertersen et al, 2008) and with normal nucleus (Oliveira et al, 2008). However, HAB selection improved the level of spermatozoa with denatured DNA (Pertersen et al, 2008). HAB selection also did not positively affect the fertilization rate after ICSI (Bacer Kermavner et al, 2008) and was not usable as a predictive value for the outcome of IVF/ICSI treatment (Nijs et al, 2008). HAB did have a positive effect on the further embryo development (Bacer Kermavner et al, 2008).

Annexin V MACS Separation

Although the presence of apoptosis in ejaculated sperm compared with somatic cells still remains to be demonstrated (Sakkas et al, 1999; Sakkas et al, 2002), ejaculates from infertility patients are of low quality as measured by standard semen parameters and the presence of apoptosis-like characteristics e.g., activated caspases, externalization of phosphatidylserine (PS) at the outer plasma membrane, DNA fragmentation and MMP disruption (Comhaire et al, 1988; Glander and Schaller, 1999; Grunewald et al, 2005; Oehninger et al, 2003; Paasch et al, 2003b).

Externalization of PS is the main apoptotic event detectable at the sperm surface, although it usually is present only on the inner leaflet of the sperm plasma membrane (Oosterhuis and Vermes, 2004; Vermes et al, 1995). Annexin V is a phospholipids-binding protein that has high affinity for PS and lacks the ability to pass through an intact sperm membrane (van Heerde et al, 1995). Therefore, annexin V binding to spermatozoa may be used to label sperm that have compromised membrane integrity and that are less capable to fertilize eggs (Glander and Schaller, 1999).

Annexin V-conjugated super-paramagnetic microbeads can effectively separate non-apoptotic spermatozoa from those with deteriorated plasma membranes based on the externalization of PS using magnetic-activated cell sorting (MACS). MACS separation of sperm yields two fractions: annexin V-negative (intact membranes, nonapoptotic) and annexin V-positive (externalized PS, apoptotic) which is retained in the magnetic field (Grunewald et al, 2001; Paasch et al, 2003b). Sperm preparation that combines MACS with double-density centrifugation provides spermatozoa of higher quality in terms of motility, viability and apoptosis indices (caspase activation, MMP disruption and DNA fragmentation) compared with other conventional sperm preparation methods (Said et al, 2005b). Furthermore, sperm prepared according to this protocol showed improved ability to fertilize eggs using the hamster oocyte penetration assay (Said et al, 2005a). Current studies support the conclusion that MACS improves semen quality and cryosurvival rates and increases the oocyte penetration potential (Said et al, 2008).

Recently we have demonstrated that the annexin-negative fraction after MACS separation has consistently lower cellular iron content than the control fraction. We assume that this is evidence that MACS does not only depletes the cells labelled with magnetic beats, but also removes cells with a known high intrinsic magnetic susceptibility.

Annexin V Molecular Glass Wool Separation

Although integrating annexin V-MACS into standard semen preparation protocols offers a potentially major advantage, the possibility of an accidental transmission of super-paramagnetic microbeads into eggs cannot be fully excluded. A sperm preparation system using the binding properties of annexin V by avoiding free flotation of super-paramagnetic microbeads in a liquid phase buffer might help avoid side effects. The annexin V glass filtration technique is a promising step towards the development of a molecular-based preparation system with an enhanced capability for selecting vital spermatozoa with superior fertilizing capacity. We recently demonstrated the feasibility of combining the classical glass wool filtration method with phosphatidylserine-binding properties of annexin V and a solid-phase molecular filtration system. We investigated the apoptosis markers of sperm following simple wash, glass wool filtration, molecular glass wool filtration and MACS. We demonstrated that the application of annexin V glass wool may improve the outcome of assisted reproductive techniques. Our data support the highly efficient filtration capacity of a solid-phase annexin V-coated glass wool filter (Grunewald et al, 2007).

Further attempts to bind the annexin V on glass beads and separate the sperm cells by MECS did not show satisfying results. The motility as well as the caspase activation showed inferior data after the separation.

CONCLUSION

An estimated 2-4% of new births in developed countries now involve assisted conception techniques. While this technology has revolutionized the treatment of infertile couples, classic sperm preparation techniques still require improvements in evaluation, and advanced and molecular selection strategies are still in the developmental stages.

Insufficient evidence exists to recommend any of the classical preparation techniques. Large, high quality, randomized, controlled trials, comparing the effectiveness of gradient, swim-up, wash and a centrifugation techniques on clinical outcome are lacking. Results from studies comparing semen parameters may suggest a preference for gradient techniques, but firm conclusions cannot be drawn, and the limitations should be taken into consideration (Boomsma et al, 2004).

Safety of the sperm selection methods is another area for improvement. The presence of DNA damage in the fertilizing spermatozoon is believed to be responsible for early pregnancy loss and birth defects (Ainsworth et al, 2005). Therefore, an urgent need exists for optimized procedures for isolating spermatozoa for ART that maintain full functional competence by avoiding extrinsic harm due to ROS production (Aitken et al, 1998; Zini et al, 2000).

The latest developments in advanced and molecular sperm selection strategies open a new field to develop customized sperm preparation techniques according to the primary quality of the ejaculate and the method of ART to be used.

BIBLIOGRAPHY

1. Agarwal A, Manglona A, Loughlin KR. Improvement in semen quality and sperm fertilizing ability after filtration through the L4 membrane: comparison of results with swim up technique. J Urol 1992;147:1539-41.
2. Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. Hum Reprod 2005;20:2261-70.
3. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998;59:1037-46.
4. Al Hasani S, Kupker W, Baschat AA, Sturm R, Bauer O, Diedrich C, Diedrich K. Mini-swim-up: a new technique of sperm preparation for intracytoplasmic sperm injection. J Assist Reprod Genet 1995;12:428-33.
5. Babbo CJ, Hecht BR, Jeyendran RS. Increased recovery of swim-up spermatozoa by application of "antigravitational" centrifugation 1. Fertil Steril 1999;72: 556-58.
6. Bacer Kermavner L, Virant-Klun I, Vrtacnik-Bokal E, Tomazevic T, Meden-Vrtovec H. Sperm selection with hyaluronan for ICSI procedures and blastocyst development. Hum Reprod 2008;23:i158. Ref Type: Abstract
7. Barroso G, Taylor S, Morshedi M, Manzur F, Gavino F, Oehninger S. Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations. Fertil Steril 2006;85:149-54.

8. Bartoov B, Berkovitz A, Eltes F, Kogosovsky A, Yagoda A, Lederman H, Artzi S, Gross M, Barak Y. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 2003;80:1413-19.
9. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J Androl 2002;23:1-8.
10. Berger T, Marrs RP, Moyer DL. Comparison of techniques for selection of motile spermatozoa. Fertil Steril 1985;43:268-73.
11. Bolton VN, Braude PR. Preparation of human spermatozoa for in vitro fertilization by isopycnic centrifugation on self-generating density gradients. Arch Androl 1984;13:167-76.
12. Bongso A, Ng SC, Mok H, Lim MN, Teo HL, Wong PC, S Ratnam. Improved sperm concentration, motility, and fertilization rates following Ficoll treatment of sperm in a human in vitro fertilization program. Fertil Steril 1989;51:850-54.
13. Boomsma CM, Heineman MJ, Cohlen BJ, Farquhar C. Semen preparation techniques for intrauterine insemination. Cochrane Database Syst Rev 2004;CD004507.
14. Bostan A, Vannin AS, Emiliani S, Liesnard C, Englert Y. Development and evaluation of single sperm washing (SSW) for decontamination in extreme oligospermic HIV positive patients. Human Reproduction 2008;23:i85. Ref Type: Abstract.
15. Calamera JC, Quiros MC, Brugo S, Nicholson RF. Comparison between swim-up and glass bead column techniques for the separation of human spermatozoa. Andrologia 1991;23:259-61.
16. Chen MJ, Bongso A. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. Hum Reprod 1999;14:759-64.
17. Chijioke PC, Crocker PR, Gilliam M, Owens MD, Pearson RM. Importance of filter structure for the trans-membrane migration studies of sperm motility. Hum Reprod 1988;3:241-44.
18. Claassens OE, Menkveld R, Harrison KL. Evaluation of three substitutes for Percoll in sperm isolation by density gradient centrifugation. Hum Reprod 1998;13:3139-43.
19. Coetzee K, Erasmus EL, Kruger TF, Menkveld R, Lombard CJ. Glass wool filter preparation of cryopreserved spermatozoa. Andrologia 1994;26:33-34.
20. Comhaire FH, Vermeulen L, HInting A, Schoonjans F. Accuracy of sperm characteristics in predicting the in vitro fertilizing capacity of semen. J *In Vitro* Fert Embryo Transf 1988;5:326-31.
21. Counsel M, Bellinge R, Burton P. Vitality of oligozoospermic semen samples is improved by both swim-up and density gradient centrifugation before cryopreservation. J Assist Reprod Genet 2004;21:137-42.
22. Daya S, RB Gwatkin. Improvement in semen quality using glass bead column. Arch Androl 1987;18:241-44.
23. Daya S, Gwatkin RB, Bissessar H. Separation of motile human spermatozoa by means of a glass bead column. Gamete Res 1987;17:375-80.
24. Dominguez LA, MH Burgos, MW Fornes. Morphometrical comparison of human spermatozoa obtained from semen and swim-up methodology. Andrologia 1999;31:23-26.
25. Drobnis EZ, Zhong CQ, Overstreet JW. Separation of cryopreserved human semen using Sephadex columns, washing, or Percoll gradients. J Androl 1991;12:201-08.
26. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization *in vitro* of human oocytes matured in vitro. Nature 1969;221:632-35.
27. Ford WC, McLaughlin EA, Prior SM, Rees JM, Wardle PG, Hull MG. The yield, motility and performance in the hamster egg test of human spermatozoa prepared from cryopreserved semen by four different methods. Hum Reprod 1992;7:654-59.
28. Franco Jr JG, Baruffi RLR, Mauri ALM, Petersen CG, Felipe V, Vagini LD, Oliveira JBA. Large nuclear vacuoles are indicative of significant DNA damage in human spermatozoa:implication for ICSI. Hum Reprod 2008;23:i114-i115. Ref Type: Abstract
29. Gabriel LK, Vawda AI. Preparation of human sperm for assisted conception: a comparative study. Arch Androl 1993;30:1-6.
30. Gellert-Mortimer ST, Clarke GN, Baker HW, Hyne RV, Johnston WI. Evaluation of Nycodenz and Percoll density gradients for the selection of motile human spermatozoa. Fertil Steril 1988;49:335-41.
31. Glander HJ. Effektivität der Methoden für die Spermienkollektion, -separation und -konzentrierung. Hautnah derm 1993;9:52-60.
32. Glander HJ, Schaller J. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Hum Reprod 1999;5:109-15.
33. Glander HJ, Schaller J, Ladusch M. A simple method for separating fresh and cryopreserved human sperm using dextran-visotrast density gradient centrifugation. Zentralbl Gynakol 1990;112:91-97.
34. Gorus FK, Pipeleers DG. A rapid method for the fractionation of human spermatozoa according to their progressive motility. Fertil Steril 1981;35:662-65.
35. Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. Hum Reprod 2005;20:2590-94.
36. Grunewald S, Miska W, Miska G, Rasch M, Reinhardt M, Glander HJ, Paasch U. Molecular glass wool filtration as a new tool for sperm preparation. Hum Reprod 2007;22:1405-12.
37. Grunewald S, Paasch U, Glander HJ. Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. Cell and Tissue Banking 2001;2:127-33.

38. Grunewald S, Paasch U, Wuendrich K, Glander HJ. Sperm caspases become more activated in infertility patients than in healthy donors during cryopreservation. Arch Androl 2005;51:449-60.
39. Hammadeh ME, Zavos PM, Rosenbaum P, Schmidt W. Comparison between the quality and function of sperm after semen processing with two different methods. Asian J Androl 2001;3:125-30.
40. Harrison RA. A highly efficient method for washing mammalian spermatozoa. J Reprod Fertil 1976;48:347-53.
41. Hauser R, Homonnai ZT, Paz GF, Yavetz H, Amit A, Lessing JB, Peyser MR, Yogev L. Migration sedimentation technique as a predictive test for the fertilizing capacity of spermatozoa in an *in-vitro* fertilization programme. Int J Androl 1992;15:498-503.
42. Henkel R, Franken DR, Lombard CJ, Schill WB. Selective capacity of glass-wool filtration for the separation of human spermatozoa with condensed chromatin: A possible therapeutic modality for male-factor cases? Journal of Assisted Reproduction and Genetics. 1994a;11:395-400.
43. Henkel R, Schill WB. Sperm separation in patients with urogenital infections. Andrologia 1998;30 Suppl 1:91-97.
44. Henkel RR, Franken DR, Lombard CJ, Schill WB. Selective capacity of glass-wool filtration for the separation of human spermatozoa with condensed chromatin: A possible therapeutic modality for male-factor cases? J Assist Reprod Genet 1994b;11:395-400.
45. Henkel RR, Schill WB. Sperm preparation for ART. Reprod Biol Endocrinol 2003;1:108.
46. Holmgren WJ, Jeyendran RS. Synergistic effect of TEST—yolk buffer treatment and glass wool filtration of spermatozoa on the outcome of the hamster oocyte penetration assay. Hum Reprod 1993;8:425-27.
47. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril 2003;79 Suppl 3:1616-24.
48. Huszar G, Ozkavukcu S, Jakab A, Celik-Ozenci C, Sati GL, Cayli S. Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. Curr Opin Obstet Gynecol 2006;18:260-67.
49. Huszar G, Willetts M, Corrales M. Hyaluronic acid (Sperm Select) improves retention of sperm motility and velocity in normospermic and oligospermic specimens. Fertil Steril 1990;54:1127-34.
50. Jakab A, Kovacs T, Zavaczki Z, Borsos A, Bray-Ward P, Ward D, Huszar G. Efficacy of the swim-up method in eliminating sperm with diminished maturity and aneuploidy. Hum Reprod 2003;18:1481-88.
51. Jameel T. Sperm swim-up: a simple and effective technique of semen processing for intrauterine insemination. J Pak Med Assoc 2008;58:71-74.
52. Johnson DE, Confino E, Jeyendran RS. Glass wool column filtration versus mini-Percoll gradient for processing poor quality semen samples. Fertil Steril 1996;66:459-62.
53. Kato S, Hanabusa H, Kaneko S, Takakuwa K, Suzuki M, Kuji N, Jinno M, Tanaka R, Kojima K, Iwashita M, Yoshimura Y, Tanaka K. Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1. AIDS 2006;20:967-73.
54. Klinc P, Kosec M, Majdic G. Freezability of equine semen after glass beads column separation. Equine Vet J 2005;37:43-47.
55. Larson KL, Brannian JD, Timm BK, Jost LK, Evenson DP. Density gradient centrifugation and glass wool filtration of semen remove spermatozoa with damaged chromatin structure. Hum Reprod 1999a;14:2015-19.
56. Larson KL, Brannian JD, Timm BK, Jost LK, Evenson DP. Density gradient centrifugation and glass wool filtration of semen remove spermatozoa with damaged chromatin structure. Hum Reprod 1999b;14:2015-19.
57. Lechtzin N, Garside W, Heyner S, Hillman N. Glass-bead column separation of motile and nonmotile human spermatozoa. J *In Vitro* Fert Embryo Transf 1991;8:96-100.
58. Makkar G, Ng HY, Yeung SB, Ho PC. Comparison of two colloidal silica-based sperm separation media with a non-silica-based medium. Fertil Steril 1999;72:796-802.
59. Mashiach R, Fisch B, Eltes F, Tadir Y, Ovadia J, Bartoov B. The relationship between sperm ultrastructural features and fertilizing capacity in vitro. Fertil Steril 1992;57:1052-57.
60. McCann CT, Chantler E. Properties of sperm separated using Percoll and IxaPrep density gradients. A comparison made using CASA, longevity, morphology and the acrosome reaction. Int J Androl 2000;23:205-09.
61. Mortimer D, Mortimer ST. Methods of sperm preparation for assisted reproduction. Ann Acad Med Singapore 1992;21:517-24.
62. Nijs M, Creemers E, Cox A, Vanheusden E, Franssen K, Ombelet W. Relationship between hyaluronic acid binding assay and outcome in ART: A prospective randomised study. Hum Reprod 2008;23:i105-i106. Ref Type: Abstract
63. Oehninger S, Morshedi M, Weng SL, Taylor S, Duran H, Beebe S. Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. Reproductive Biomedicine Online. 2003;7:469-76.
64. Ohashi K, Saji F, Wakimoto A, Kato M, Tsutsui T, O Tanizawa. Preparation of oligozoospermic and/or asthenozoospermic semen for intrauterine insemination using the SpermPrep semen filtration column. Fertil Steril 1992;57:866-70.
65. Oliveira JBA, Peterson CG, Mauri AL, Masso FC, Felipe V, Baruffi RLR, Franco Jr JG. Hyaluronic acid binding is not efficient to select motile spermatozoa with normal nuclear morphology. Hum Reprod 2008;23:i104-i105. Ref Type: Abstract
66. Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, Evenson D, Thomas AJ Jr, Alvarez JG. Characterization of subsets of human spermatozoa

at different stages of maturation: Implications in the diagnosis and treatment of male infertility. Hum Reprod 2001;16:1912-21.
67. Oosterhuis GJ, and I Vermes. Apoptosis in human ejaculated spermatozoa. J Biol Regul Homeost Agents 2004;18:115-19.
68. Öztürk Turhan N, Pekel A, Arikan Onaran Y, Iltemir Duvan C, Bayrak O, Kafali H. One wash or two washes:effects of sperm wash processing by density gradient centrifugation on intrauterine insemination outcomes. Hum Reprod 2008;23:i119-i120. Ref Type: Abstract.
69. Paasch U, Agarwal A, Gupta AK, Sharma RK, Grunewald S, Thomas AJ, Jr., Glander HJ. Apoptosis signal transduction and the maturity status of human spermatozoa. Ann. NY. Acad Sci 2003a;1010:486-89.
70. Paasch U, Grunewald S, Fitzl G, Glander HJ. Deterioration of plasma membrane is associated with activation of caspases in human spermatozoa. J Androl 2003b;24:246-52.
71. Paulson JD, Polakoski KL. A glass wool column procedure for removing extraneous material from the human ejaculate. Fertil Steril 1977;28:178-81.
72. Paulson JD, KL Polakoski, Leto S. Further characterization of glass wool column filtration of human semen. Fertil Steril 1979;32:125-26.
73. Pertersen CG, Vagnini L, Baruffi RLR, Mauri AL, Ricci J, Oliviera JG, Franco Jr JG. Hyaluronic acid binding assay is not efficient to select spermatozoa without DNA damage. Human Reprod 2008;23:i87. Ref Type: Abstract
74. Pharmacia Biotech I. Important notice: Percoll® NOT to be used in Assisted Reproduction Technologies in Humans. <None Specified>. 1996.
75. Pickering SJ, Fleming TP, Braude PR, Bolton VN, Gresham GA. Are human spermatozoa separated on a Percoll density gradient safe for therapeutic use? Fertil Steril 1989;51:1024-29.
76. Pousette A, Akerlof E, Rosenborg L, Fredricsson B. Increase in progressive motility and improved morphology of human spermatozoa following their migration through Percoll gradients. Int J Androl 1986;9:1-13.
77. Rasch M, Miska W, Grunewald S, Glander HJ, Paasch U. Molecular classification separated by glass wool filtration. p. 1-4. In S.-C.Kim, J.A.Grootegoed, and H.Chemes (ed.) 8th International Congress of Andrology. Medimond, Medimond, 2005.
78. Rhemrev J, Jeyendran RS, Vermeiden JP, Zaneveld LJ. Human sperm selection by glass wool filtration and two-layer, discontinuous Percoll gradient centrifugation. Fertil Steril 1989;51:685-90.
79. Said TM, Agarwal A, Grunewald S, Rasch M, Baumann T, Kriegel C, Li L, Glander HJ, Thomas Jr AJ, Paasch U. Selection of Non-Apoptotic Spermatozoa as a New Tool for Enhancing Assisted Reproduction Outcomes: An In Vitro Model Biol Reprod 2005a;74:530-37.
80. Said TM, Agarwal A, Zborowski M, Grunewald S, Glander HJ, Paasch U. Utility of magnetic cell separation as a molecular sperm preparation technique. J Androl. 2008;29:134-42.
81. Said TM, Grunewald S, Paasch U, Glander HJ, Liang L, Agarwal A. Advantage of combining magnetic cell separation with sperm preparation technique. RBM online. 2005b;10:740-46.
82. Sakkas D, Mariethoz E, St John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp. Cell Res 1999;251:350-55.
83. Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod 2002;66:1061-67.
84. Sanchez R, Concha M, Ichikawa T, Henkel R, Schill WB. Glass wool filtration reduces reactive oxygen species by elimination of leukocytes in oligozoospermic patients with leukocytospermia. J Assist Reprod Genet 1996;13:489-94.
85. Savasi V, Parrilla B, Ratti M, Ferrazzi E. Reproductive assistance in HIV-1 discordant couples. Curr Opin Obstet Gynecol 2008;20:205-10.
86. Scott RT, Oehninger Jr SC, Menkveld R, Veeck LL, Acosta AA. Critical assessment of sperm morphology before and after double wash swim-up preparation for in vitro fertilization. Arch Androl 1989;23:125-29.
87. Sherman JK, Paulson JD, Liu KC. Effect of glass wool filtration on ultrastructure of human spermatozoa. Fertil Steril 1981;36:643-47.
88. Sills ES, Wittkowski KM, Tucker MJ, Perloe M, Kaplan CR, Palermo GD. Comparison of centrifugation- and noncentrifugation-based techniques for recovery of motile human sperm in assisted reproduction. Arch Androl 2002;48:141-45.
89. Society for Assisted Reproductive Technology, andAmerican 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;81:1207-20.
90. Soderlund B, Lundin K. The use of silane-coated silica particles for density gradient centrifugation in in-vitro fertilization. Hum Reprod 2000;15:857-60.
91. Srisombut C, Morshedi M, Lin MH, Nassar A, Oehninger S. Comparison of various methods of processing human cryopreserved- thawed semen samples. Hum Reprod 1998;13:2151-57.
92. Tea NT, Jondet M, Scholler R. 1983. A "migration – gravity – sedimentation" method for collecting human motile spermatozoa. In Harrison F (ed.) World congress on fertility and sterility. 26 th June—1st July 1883. Proceedings of the Congress.
93. Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human

spermatozoa during sperm preparation: protective significance of seminal plasma. Mol Hum Reprod 1998;4:439-45.
94. Van Den BM, Revelard P, Bertrand E, Biramane J, Vanin AS, Englert Y. Glass wool column filtration, an advantageous way of preparing semen samples for intracytoplasmic sperm injection: an auto-controlled randomized study. Hum Reprod 1997a;12:509-13.
95. Van Den BM, Revelard P, Bertrand E, Biramane J, Vanin AS, Englert Y. Glass wool column filtration, an advantageous way of preparing semen samples for intracytoplasmic sperm injection: an auto-controlled randomized study. Hum Reprod 1997b;12:509-13.
96. Van der Ven HH, Jeyendran RS, Al Hasani S, Tunnerhoff A, Hoebbel K, Diedrich K, Krebs D, Perez-Pelaez M. Glass wool column filtration of human semen: relation to swim-up procedure and outcome of IVF. Hum Reprod 1988;3:85-88.
97. Van Heerde WL, de Groot PG, Reutelingsperger CP. The complexity of the phospholipid binding protein Annexin V. Thromb Haemost 1995;73:172-79.
98. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger CP. A novel assay for apoptosis: Flow cytometric detection of phosphatidylserine expression of early apoptotic cells using fluorescein labelled Annexin V J Immunol Methods 1995;184:39-51.
99. Wikland M, Wik O, Steen Y, Qvist K, Soderlund B, Janson PO. A self-migration method for preparation of sperm for in-vitro fertilization. Hum Reprod 1987;2:191-95.
100. Wu TP, Huang BM, Tsai HC, Lui MC, Liu MY. Effects of nitric oxide on human spermatozoa activity, fertilization and mouse embryonic development. Arch Androl 2004;50:173-79.
101. Yavetz H, Hauser R, Homonnai ZT, Paz GF, Lessing JB, Amit A, Yogev I. Separation of sperm cells by sedimentation technique is not suitable for in vitro fertilization purposes. Andrologia 1996;28:3-6.
102. Yener C, Mathur S, Parent B. Comparison of two sperm preparation techniques using automated sperm motion analysis: migration sedimentation versus swim-up. Arch Androl 1990;25:17-20.
103. Younglai EV, Holt D, Brown P, Jurisicova A, Casper RF. Sperm swim-up techniques and DNA fragmentation. Hum Reprod 2001;16:1950-53.
104. Zavos PM, Centola GM. Methods of semen preparation for intrauterine insemination and subsequent pregnancy rates. Tohoku J Exp Med 1992;168:583-90.
105. Zini A, Nam RK, Mak V, Phang D, Jarvi K. Influence of initial semen quality on the integrity of human sperm DNA following semen processing. Fertil Steril 2000;74:824-27.

CHAPTER 19

Preparation of Semen Sample Infected with HIV for ART

MS Srinivas, Kamini A Rao

INTRODUCTION

The human immunodeficiency virus (HIV) was discovered in the 1980s and natural history of the HIV has been studied extensively. This is a disease originally described as affecting male homosexuals but today heterosexual is the leading mode of transmission in many countries.[1] Majority of the countries around the world do not have access to antiviral drugs due to poverty. The spread of the infection has slowed down in the developed countries. The HIV still mainly affects young adults, but prognosis has been changed considerably by the remarkable therapeutic advances made in recent years. Many HIV infected people are most of the time in a good general state and they can make plans for the future again.[2] These plans quite logically in young couples may include the desire to have child.

Medically assisted reproductive team have always been confronted with request for assistance from seropositive people. These request are now becoming increasingly numerous and insistent.[3] The general advice given to couple with seropositive not to plan for child because both the couple carrying the virus and there is risk of horizontal transmission to the other partner if the man is carrying the virus and vertical transmission to the child if the woman is the one affected. Since, 1980 the artificial insemination with donor semen sample was in practice for women whose partner is seropositive. This method really helps the couple to become parents with no risk of the disease being transmitted to either the mother or the child.[4] A number of practitioners are reassessing their position in this field. The survey carried out among heads of gynecological departments in France revels that 47% of them are in agreement with taking charge locally of seropositive women with infertility problems. Forty-two percent of them say that they are prepared to conduct stimulation of ovulation in seropositive women.[5]

In the year 2002 American College of Obstetricians and Gynecologists (ACOG) and the American Society for Reproductive Medicine (ASRM) recommended sperm washing to be offered to HIV- serodiscordant couples as a standard of care.[6] Semen sample of serodiscordent couple washed used for IUI as well as IVF/ICSI procedure and many babies born.

India has a population of one billion, around half of whom are adults in the sexually active age group. The first AIDS case in India was detected in 1986; since then HIV infection has been reported in all states and union territories. There is a need to evolve standard care for seropositive couples. Today, it is possible to assists these couple by using medically assisted reproductive technologies.

Medical Assisted Reproduction for Seropositive Cases

Semen used in artificial insemination can transmit infection by the HIV-1 virus. The first case of infection in artificial insemination with a Donor (AID) in Australia also in Canada and USA have been reported.[7] To avoid this transmission the semen donors must be screened and frozen semen sample to be placed in quartine for a period of 6 months. Donor semen sample is used for the AID only if a new test carried out on the Donor is negative.[8]

The presence of viral particles has been demonstrated in the seminal plasma. It exists free form or in the cellular components in the intracellular form.[9] Presence of viral particles has been confirmed through an autopsy in the white cells of the tissues of the entire male genital tract.[10] The relationship between the viral concentration in the plasma and the semen is not constant and there is no agreement in the literature today the extent of variation of the concentration in viral particles measured on samples taken successively from the same patient considered to be clinically stable. On the other hand, it is clearly established that methods of preparing the semen in which the seminal fluid and other cellular elements are separated from the spermatozoa by washing may reduce the viral load up to a level undetectable by the most sensitive techniques[11] (PCR-RNA and PCR-DNA). It is known that antiviral treatments that are very active at the plasmic level reduce the viral load in the spermatozoa. All these data have lead to use assisted reproduction techniques allowing serodifferent couples in which the man is carrying the virus to have children using the man's own sperm.

Dr Semprini,[12] the pioneer of the use of intraconjugal insemination with washed sperm, reported birth of 350 children without viral contamination by various ART procedures which includes Intrauterine Insemination (IUI), *In vitro* Fertilization and Embryo Transfer (IVF-ET) and Intracytoplasmic Sperm Injection (ICSI).

For better help to these couple through assisted reproduction, there should be a appropriate facilities. It is necessary to keep separate facilities for treating the biological liquids of these patients and to develop special safety procedures both for the sake of the staff and for the prevention of interpatient contamination. Logical approach must be to build one separate laboratory for IVF, ICSI and dedicated area for semen preparation of seropositive patients.

Effect of HIV on Semen Parameters

Semen samples are obtained by masturbation into a sterile plastic containers with 3 days of abstinence. Analysis should be carried out in a separate laboratory designated for treatment of infectious disease. While doing analysis powder free Latex gloves, mouth-face mask, plastic apron and goggles must be worn all the times. Sample is allowed to liquefy at room temperature for 30 minutes prior to analysis. Semen examination includes three important characteristics such as physical, microscopic features and optinal test. The various semen parameters is to be assessed as outlined by the World Health Organization 1999 criteria.[13]

JDM Nicopoullos et al[14] demonstrated that various parameters like total ejaculate volume, sperm concentration, total count motility, progressive motility and morphology was impaired in HIV men, when compare to control group. The first study to present data on sperm characteristics in HIV+ men found no difference in any parameters between their small cohort and control group. In a slightly larger group of HIV+ men demonstrated significantly lower sperm motility, which includes percent motility, motile sperm density and total motile count per ejaculate. In the largest group of HIV+ men found to have significantly lower ejaculate volume, sperm concentration and motility. Abstinence delay can also impact on sperm parameters. It could be HIV+ men may have longer abstinence periods due to the effect of illness on libido, fear of transmission and cultural differences in frequency of masturbation. Dulioust et al[15] 2002 found impaired parameters in their cohort before and after matching for abstinence delay.

HIV infected sperm samples were correlated with markers. There is significant correlation between CD4 cell count and ejaculate volume, sperm concentration, total sperm count, sperm motility, progressive motility and sperm morphology. The effect of CD4 count on the postpreparation samples used for IUI was assessed, found significant effect of CD4 count on sperm concentration, morphology and total motility concentration of spermatozoa inseminated. Due to sub-optimal semen processing researcher proposed ICSI as the method of choice for assisted reproduction regardless of semen parameters.[16]

Semen Processing

The objective of semen preparation for various assisted reproductive technology is to obtain good motile, count, and morphology to enhance the fertilization rate. Semen sample with normal parameters will always yield good motile spermatozoa which is necessary for IVF. Sub-fertile samples it is difficulty to get required sperm count after processing in such instances intracytoplasmic sperm injection (ICSI) is the only solution to overcome the male infertility. For ICSI, live sperms with ability to activate oocyte and for pronucleus are necessary, but morphology, motility and acrosome status are generally not important.

It is prerequisite to remove the seminal plasma before the procedure because seminal plasma contains decapacitation factors and extraneous cells, and degenerating sperm that may produce agent capable of damaging the sperm.

Damage to the sperm by dilution, temperature change, centrifugation, and exposure to potentially toxic material must be minimized. Minimizing centrifugation in the absence of seminal plasma, and separating the live motile sperm from the dead sperm and debris early in the procedure should limit oxidative damage cause by free oxygen radicals released from leukocytes or abnormal spermatozoa. The gradient centrifugation and swim-up may produce higher yields of good-quality sperms. After the introduction of micromanipulation technique the need for special preparation technique receded.

Sperm Washing for HIV-1 Serodiscordant Couple

- Semen sample is generally collected by masturbation into a sterile container after 4-7 days of sexual abstinence.
- Allow the sample to liquefy at room temperature for 10-20 minutes.
- After liquefaction semen parameters is to be assessed using World Health Organization (WHO) criteria
- Take 2 ml of 80% and 40% in a sterile tube respectively.
- Semen processing can also be performed using triple density gradient
- Overlay the liquefied semen sample on top of 40% gradient gently without disturbing the gradient
- Centrifuge at 1800 rpm for 20 minutes
- After centrifugation discard the supernatant and recovered sperm pellet is resuspended with fresh culture medium.
- Resuspended sperm pellet centrifuged for another 10 minutes at 1800 rpm
- After the second wash, discard the supernatant and over- lay the pellet with 1 ml of culture medium and incubate for 1 hour.

The above steps for sperm processing for assisted reproductive technologies is same as we do routinely in the andrology/IVF laboratory. After the swim-up supernatant with sperm suspension is recovered and an aliquot of this is checked for detectable HIV RNA. Nucleic acid extraction and HIV-1 RNA quantification is performed using realtime PCR assay.

PCR Technique to Detect Viral Load

- Extract the Nucleic acid from the washed spermatozoa using Nuclisence method (Organon teknika).
- Prepare two extraction one from the spermatozoa sample and another with the spermatozoa samples after addition of HIV RNA obtained from HIV-infected plasma to detect the presence of transcription or amplification inhibitors after the nucleic acid extraction procedure.
- Use the extracted samples for two HIV RNA transcription to detect genes from the gag and pol region, followed by a nested DNA amplification.
- Same sample is used to amplify HIV proviral DNA by a nested amplification to detect both genes.
- Second extraction run in parallel with added HIV RNA before nucleic acid extraction is used as positive control to detect the presence of inhibitors of the transcription of amplification.

Processed semen sample is stored and an aliquot of this is used to find out detectable HIV RNA. If the PCR test for HIV is negative then only sample is used for IUI and IVF/ICSI procedures.

Nicolous Garido et al demonstrated that there is no relevence of the density gradient or swim-up volume in the results. They found no difference between the treatment practiced on the samples that yielded positive results after the sperm wash and those that were negative after the nPCR determination. Regarding the correlation between semen parameters, blood CD4 levels and viral load levels, there is clear confirmation as the disease evolved in time, sperm motility is not impaired. This is in contradiction to the results found by other groups, in which the total volume is diminished as long as the infection progresses in time.[15]

There is a positive correlation between the CD4 cell count in the blood with ability of spermatozoa to undergo capacitation. This indicates that an adequate immune status favors the production of spermatozoa which are able to trespass two consecutive semen prepartion.[17]

Receiving antiretroviral treatment seems not to impair spermatogenesis in HIV-seropositve males since the results of the semen analysis were comparable. Studies favoring[18] or against this hypothesis are available in the literature. Moreover antiretroviral treatment had no influence either on the results of the sperm wash or on the semen characteristics after the sperm wash.

THE ETHICAL ISSUES

The ethical issues involved in counseling in seropositive cases is developed only up to certain point. Apart from the necessary reminder of the general rules of medical ethics that are applicable, namely the obligation to provide medical assistance and to treat patients carrying the HIV virus[19] with out abandoning them, it is understood that the first role of the practitioner in this field is that of information and counseling detailing the implication of the disease for sexual life and reproduction, as well as promote safe sex. When there is a desire for child, the first approach consists examining it openly with patients, remembering that the dynamic of this desire can be different for each member of the couple. It is necessary to work with patients on the meaning of this desire within the particular context of one of the partners being seropositive and to take account not only of the risk of transmission to the child but also of the difficulty of combining being a parent with the constraints of their illness.[4] Their feelings in relation to expressing their own needs in the face of the child's needs and risk of the child becoming an orphan must also be discussed.[20] Health professionals is not really confronted with an ethical dilemma, they plays a role of both the patients' private adviser and, at the same time, does every thing in his power to avoid the risk of the birth of a child infected by the AIDS virus. It is not possible to provide assistance in reproduction and maintain a firm line of advice against pregnancy. However, lack of assistance in the desire for pregnancy leads a number of couples to chose to have unprotected sexual intercourse.[21] These couples also tend to distance themselves from the medical structures by which they feel rejected.[22] It therefore seems that what must prevail in the medical decision is a balance the importance of the message advising against pregnancy and the benefit for patients of being assisted in their plans to have a child.

SUMMARY

The HIV-1 infected patients now have the possibility to become fathers, avoiding viral transmission to the mother and future child. Sperm parameters are not significantly different from those that the World Health Organization considered to be normal. It is not possible to detect a parameter that could help us in the prediction of the sperm wash results, which remain positive in 10% of the samples. Low CD4 counts together with a long time of evolution of the disease are relevent for the sample, but insignificant for the whole treatment. Knowing all these results, patients must be counseled accordingly before the initiation of a treatment.

REFERENCES

1. Public health laboratsory service AIDS and STD Centre communicable Disease Surveilence Centre, and Scottish Centre for infection and Environmental Health (2002) Unpublished Quarterly Surveilence Tables No. 57.
2. Yvon Englert, Jean-paul Van Vooren, et al., 2001 ART in HIV infected couples Has the time come for a change of attitude? Hum Reprod 2001;Vol.16, No.7 pp, 1309-15.
3. Hammamah S., Girard M, et al. Couples serodifferents: la demande aupres des centres biologiques d'assistance medicale a la procreation. Communication au congres Le desire d'enfant chez les couples VIH serodifferents Toulson 2000;25-27.
4. Delvigne A, Gustin ML, et al. Le sida: Indication d'insemination artificiele avec sperme de donner anonyme? J Gynecol Obstet Bio Reprod 1990;19:751-58.
5. Bongin A, Durand-Reville M, et al. La femme VIH seropositive etat des liex en France. Communication au Congres Le desire d'enfant chez les couples VIH serodifferents Toulouse, Mai 2000;35-36.
6. American College of Obstetricians and Gynecologists Committee on Ethics. ACOG committee opinion. Human immunodeficency virus: ethical guidelines for obstetricians and gynecologists. Ostet Gynecol 2001;97 (Supp 1), 1-16.
7. Stewart GJ, Tyler JP, Cunningham AL, et al. Transmission of human T-cell lymphotropic **birus** type III (HTLV-III) by artificial insemination by donor. Lancet 1985;ii:581-85.
8. Barratt C, Englert Y, Gottlieb C, et al. Gamete Donation Guidelines. The Corsendonk consensus statement for the european union. Hum Reprod 1998;13, (Supp.2).
9. Mermin JH, Holodiny M, Katzenstein DA, et al. Detection of human immunodeficiency virus DNA and RNA in semen by the Polymerase Chain Reaction. J. Infect. DIS 1991;164:769.
10. Pudney J, Anderson DJ. Orchitis and human immunodeficiency virus type 1 infected cells in reproductive tissues from men with acquired immune deficiency syndrome. Am J Pathol 1991;139:149-60.
11. Meseguer M, Garrido N, Gimeno C, Remohi J, Simon C, Pellicer A. Comparison of polymerase chain reaction-dependent methods for detrermining athe presence of human immunodeficiency virus and hepatitis C virus washed sperm. Fertil Steril 2002;78,1199-202.
12. Semprini A, Vucetich A, Onela M, et al. Amp intra conjugale:Quelle strategie de prise en charge? L' experience italienne. Communication a la Journee:Le desire d' enfant chezles couples Vih serodifferents. Toulouse 2000;28-29.

13. World Health Organization (WHO) Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. Cambridge University Press, Cambridge, UK, 1992.
14. James DM, Nicopoullos Paula, Almedia A, et al. The effect of human immunodeficiency virus on sperm parameters and the outcome of intrauterine insemination following sperm washing. Hum. Reprod 2004;Vol, 19 No. 10:2289-97.
15. Dulioust E, Le Du A, Costagliola D, Guibert J, Kunstmann JM, Heard I, Jullard JC, Salmon D, Leruz-Ville M, Mandelbrot L et al. Semen alterations in HIV-1 infected men. Hum Reprod 2002;17,2112-18.
16. Loutradis D, Drakakis P, Kallianidist K, Pastoula E, Blesta R and Michalas S. Birth of two infants who were seronegative for human immunodeficiency virus type 1 (HIV-1) after intracytopplsmic sperm injection of sperm from HIV-1-seropositive men. Fertil Steril 2001;75,210-12.
17. Muller CH, Coombs RW, Krieger NJ. Effects of clinical stage and immunological status on semen analysis results in human immunodeficiency virus type 1 seropositve men. Andrologia 1998;30 (Suppl):15-22.
18. Robbins WA, Witt KL, Haseman JK, Dunson DB, Troiani L, Cohen MS Hamilton CD, Perreault SD, Libbus B, Beyler SA. Antiretroviral therapy effects on genetic and morphologic end points in lympocytes and sperm of men with human immunodeficiency virus infection. J Infect Dis 2001;184:127-35.
19. Emanuel EJ. Do physician have an obligation to treat patients with AIDS? N engl J Med 1988;318:1686-90.
20. Kass NE. Policy, ethics, and reproducive choice: Pregnancy and childbearing among HIV-infected women. Acta Paediatr 1994;400, Suppl:95-98.
21. Mandelbrot L, Heard I, Henrion-Geant E, et al. Natural conceptions in HIV-negative women with HIV-infected partners. Lancet 1997;349:850-51.
22. Nolan K. Human immunodeficiency virus infection, women, and pregnancy: ethical issues in: HIV Disease in pregnancy. Obstet. And Gynecol. Clin. North America 1990;17:651-68.

CHAPTER 20

Surgically Retrieved Sperm Samples

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ABSTRACT

Azoospermia, the absence of sperm in ejaculated semen, is the most severe form of male factor infertility, present in approximately 5% of all investigated infertile couples. The condition is currently classified as "obstructive" or "non-obstructive," although it is important to also consider the specific etiology of each individual case. Some cases of obstructive azoospermia are treatable using microsurgical reconstruction of the seminal tract (for example, vasectomy reversal). Unreconstructable obstructive azoospermia and non-obstructive azoospermia historically have been relatively untreatable conditions that required the use of donor spermatozoa for fertilization. The advent of intracytoplasmic sperm injection (ICSI), however, has transformed treatment of this type of severe male factor infertility. Sperm can be retrieved for ICSI from either the epididymis or the testis, depending on the type of azoospermia.

Different methods for recovering epididymal or testicular spermatozoa have been described, and each has its drawbacks and advantages. Percutaneous aspiration of the testis may be the method of choice in cases of irreparable obstructive azoospermia. Using a 21-gauge needle, spermatozoa may be recovered in 96% of patients. More patients undergoing fine-needle aspiration experienced less pain than expected as compared with those undergoing open biopsy. Microsurgical epididymal sperm aspiration (MESA) is the preferred method in patients with an incomplete work-up because, if indicated, a vasoepididimostomy can be performed concomitantly with a full scrotal exploration. In azoospermic patients with testicular failure, the sperm recovery rate (the chance of finding at least one spermatozoon) is around 50% after multiple open biopsies. However, fertilization rates after ICSI are significantly lower than in men with normal spermatogenesis, and complete fertilization may occur more frequently. Although the combination of testicular sperm extraction (TESE) and ICSI may be the sole treatment available for infertility because of non-obstructive azospermia, the overall rate is limited and ongoing pregnancies are obtained in < 20% of ICSI cycles. In patients with incomplete Sertoli cell-only syndrome, testicular damage may be limited by use of a selective microsurgical approach; less invasive methods such as fine-needle aspiration are not useful in these patients. Although the pregnancy rates reported after ICSI with frozen-thawed testicular spermatozoa from patients with primary testicular failure are relatively low, the recovery of testicular spermatozoa by open biopsy followed by cryopreservation may be the method of choice by which to prevent repeat surgery and pointless ovarian stimulation in the female partner.

Data from randomized trials are insufficient to recommend any particular surgical sperm retrieval techniques for either obstructive or non-obstructive azoospermia. Nonobstructive azoospermia is a difficult area to analyze as the physiology of the testis may differ significantly between individuals. Techniques are modified rapidly and there is much variation among different centers and surgeons. Using the least invasive and simplest (and thereby most cost-effective) method for surgical retrieval of sperm is logical in the absence of evidence to support more invasive or more technically difficult methods. The more invasive methods should currently be reserved for situations where sperm cannot be retrieved by a less invasive technique (such as needle aspiration of the epididymis or testis) or for evaluation in the context of a randomized trial.

INTRODUCTION

Azoospermia is no longer an obstacle for men to father their own genetic progeny. Using intracytoplasmic sperm injection (ICSI), surgically retrieved spermatozoa from the epididymis or testicle can successfully fertilize oocytes

and achieve pregnancies.[1-7] In most fertility centers, patients are classified as obstructive (OA) or non-obstructive (NOA) based on clinical features such as medical history, physical examination, testicular volume, epididymal markers, FSH and recently, inhibin-B.

Azoospermia, the absence of sperm in ejaculated semen, is the most severe form of male factor infertility and is present in approximately 5% of all investigated infertile couples.[8] Obstructive azoospermia is the result of obstruction in either the upper or lower male reproductive tract (epididymis, vas deferens, seminal vesicles or ejaculatory ducts). Sperm production may be normal (which may be verified through testicular biopsy), but the obstruction prevents the sperm from being ejaculated. Causes of obstructive azoospermia include vasectomy, congenital absence of vas deferens, scarring from past infections, and inguinal hernia or hydrocele operations. Non-obstructive azoospermia is the result of testicular failure. Sperm production is either severely impaired or non-existent, although in many cases sperm may be found and surgically extracted directly from the testicles. Causes of non-obstructive azoospermia include genetic and hormonal disorders, testicular maldescent and torsion, systemic disease (including cancer) drugs, radiation and toxins.[9-11]

Some cases of obstructive azoospermia are treatable using microsurgical reconstruction of the seminal tract (for example, vasectomy reversal). Pregnancy rates following reconstruction vary from 27-56% of cases, and results are determined by a number of factors such as the site and duration of the obstruction.[12-15] Unreconstructable obstructive azoospermia and non-obstructive azoospermia historically have been relatively untreatable conditions that required the use of donor spermatozoa for fertilization.

The advent of ICSI, however, has transformed treatment of this type of severe male factor infertility. ICSI involves injecting a single sperm into an oocyte, making it an ideal treatment for male factor infertility that would be otherwise untreatable using conventional IVF techniques, which require large numbers of sperm.[1] In the last two decades, pregnancies have been reported after ICSI with round spermatids recovered either from the ejaculate[16] or from a testis biopsy.[17] These initial successes with surgically recovered spermatozoa were followed by an explosion of alternative methods for recovering spermatozoa. Sperm can be retrieved for ICSI in a variety of ways depending on the type of azoospermia. In non-obstructive azoospermia, sperm needs to be directly obtained from the testis. Testicular sperm extraction (TESE), testicular biopsy and testicular sperm aspiration (TESA) are techniques used on men with non-obstructive azoospermia. The procedures can require multiple biopsies, sometimes in both testes. Testicular fine needle aspiration (TEFNA) is a relevantly new technique but seems to be a straight forward procedure that is well tolerated by men.[18]

In many cases of obstructive azoospermia, sperm can be retrieved from either the epididymis or the testis.[19] Men with obstructive azoospermia can undergo microsurgical epididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA) or any of the testicular sperm extraction techniques. Of the two epididymal techniques, PESA is less invasive and does not require microsurgical skills or equipment.[20]

Clear benefit is apparent with the use of ICSI for male factor infertility where sperm is present in the ejaculate.[21] Unprecedented success also has been seen in previously hopeless cases of infertility owing to azoospermia with surgically retrieved sperm for ICSI. Which of the increasing number of methods for surgical retrieval of sperm might be superior is unclear.

The importance of assessing the appropriate outcome measures is illustrated as well in this review as anywhere. Successful retrieval of sperm for ICSI (the outcome measure of many of the non-randomized trials) is simply the first step towards the achievement of the more clinically relevant outcomes - fertilization, embryo development, implantation, a clinical pregnancy, a live birth and finally, the most relevant outcome to every couple, a healthy baby without abnormality. The debate continues as to whether the non-natural selection of a sperm for ICSI could subtly increase the likelihood of 'abnormal' offspring. This question is even more pressing for testicular surgically retrieved sperm. The safety of testicular sperm compared with epididymal or ejaculated sperm with respect to abnormality in the offspring remains unclear.[22]

MICROSURGICAL EPIDIDYMAL SPERM ASPIRATION

The MESA technique adopted by us hás been described in detail previously by Tournaye's group.[23-27] The main drawback of MESA is that it is an invasive and expensive procedure requiring a basic knowledge of epididymal anatomy and microsurgical techniques.

Scrotomy is usually performed under general or loco-regional (cord block) anesthesia. An operating microscopy with 50-80 × magnification is the preferable method of choice, even though a binocular loupe may also be effective. When performed by a skilled surgeon with a proper microsurgical approach, the procedure causes minimal fibrosis and has minimal risk of creating postoperative obstructive of the epididymal tubules. The major benefit of this procedure is its diagnostic power: a full scrotal exploration can be performed concomitantly. Furthermore, the number of spermatozoa retrieved is high, which facilitates cryopreservation.[3-7, 28] Recovery of epididymal spermatozoa during a diagnostic procedure is certainly a valid option. ICSI may be performed later, and even in another center, using the frozen-thawed epididymal spermatozoa, without jeopardizing the ICSI success rate.

Percutaneous Epididymal Sperm Aspiration (PESA)

This technique is a less invasive variant of MESA. By means of a percutaneous pucture using a 19-gauge needle, epididymal spermatozoa may be aspirated blindly from the epididymis under local or loco-regional anesthesia. This technique has been described in detail by Craft's group.[29-31]

Its noninvasive character is the most important advantage of PESA. It can be performed easily on an outpatient basis, and the technique is quick and simple compared to MESA and its certainly more cost-effective. It has been argued that here may be fewer complications after PESA than after MESA.[32-36]

Although the potential for epididymal spermatozoa to be cryopreserved after PESA has been mentioned occasionally, no substantial data are described in the literature supporting this approach. It has also been stated that PESA may be repeated in the same patient several times with good results. The main criticism of the PESA technique is that blind percutaneous puncture may cause inadvertent damage to the fine epididymal structures and produce uncontrolled bleeding, causing postinterventional fibrosis. Another disadvantage is the impossibility of performing a proper diagnostic work-up and concomitant reconstructive microsurgery.

PESA Technique

Percutaneous epididymal sperm aspiration was performed on the day of ovum retrieval. All procedures were performed in our outpatient procedure room under local anesthesia. Cord block anesthesia was administered by injecting 2 mL of 1% lidocaine without adrenalin in the pampiniform plexus. The superior pole of the testicle was presented to the surgeon, who stabilizeds the epididymis carefully between the thumb and index finger. A 27-gauge needle was introduced into the proximal part of the epididymis, and a delicate suction was performed with a 1 mL syringe filled with human tubal fluid medium. The sample was replaced in the dish and assessed for the presence of sperm under light microscopy (200 × magnification). The epididymis was gently massaged to knead fluid into the tubing. Postoperatively, pressure was held on the aspiration site for 5 minutes in the operating room. A fluff compression dressing and scrotal supporter were then applied and left in place for 24 hours.

Micropuncture Technique

The micropuncture technique was developed because contamination with blood cells was believed to adversely affect fertilization rates.[37] Unfortunately the author does not report how many times excessive blood was found in the micropuncture samples or the number of repeated micropunctures that were performed, making it difficult to appraise success of the technique. Also it is unclear if the repeat procedures were performed in the MESA group. This is a possible source for bias. In essence, the trial could be considered a randomized comparison of MESA versus a 'three-intervention package' of (a) micropuncture with (b) nerve stimulation with (c) repeated micropunctures until no blood contamination occurred. Which of these 'three' interventions conferred the efficacy for the men in this group is unclear.

Micropuncture with nerve stimulation is not a technique in widespread use. Additionally this technique carries many of the disadvantages of a formal MESA approach. It is invasive requiring general anesthesia; it is a complex procedure requiring a high degree of operator skill. These features may explain why the micropuncture technique has not been widely used since this trial was published. Finally, the number of participants in each treatment group was small, so no strong conclusions can be drawn about the efficacy or the safety of these techniques.

Testicular Sperm Aspiration

Several methods have been described for percutaneous needle aspiration of the testis to obtain a histological or

cytological diagnosis of spermatogenesis or to recover spermatozoa: biopsy gun or 19, 21-gauge needle.[38-54] When 21-gauge or finer needles are used (fine needle aspiration, FNA) aspiration may be performed without any anesthesia, while the biopsy gun and 19-gauge needles require local or loco-regional anesthesia. The aspiration technique is simple and quick and is noninvasive except for biopsy gun systems or analogs.

In patients with normal spermatogenesis, a sperm recovery rate of 96% may be obtained by 21-gauge FNA. However, 21-gauge FNA may provide material suitable only for cytological assessment, while using larger needles may provide tissue cylinders that allow an accurate histopathological examination.

The aspiration procedure is assumed to be more patient-friendly because of its minimally invasive character. In a prospective study, of stress and pain assessment using a visual-analog scale method, they were higher in patients undergoing aspiration. However, when these data were expressed as proportions of patients experiencing more or less pain than anticipated, FNA had more patients experiencing less pain than expected compared with open biopsy. Some patients were indeed very anxious about the needle, which may explain the higher average stress scores in FNA patients. Furthermore, a few patients reported the FNA procedure as extremely painful, which increased the average pain score in the FNA group but did not increase the proportion of patients undergoing FNA and experiencing more pain than anticipated.

TESA Procedure

The testicle was immobilized by the surgeon by grasping the testicle with the epididymis and cord between his fingers while pulling the scrotum skin taut. The surgeon was then able to control the depth of the needle excursion, protecting the epididymis from injury. A 21-gauge butterfly needle was inserted in the superior pole of the testis and quickly advanced and pulled back multiple times toward several parts of parenchyma. The butterfly tubing was clamped adjacent to the hub of the syringe when the plunger reached 20 cc. This clamping allowed the assistant to replace the 20 cc syringe filled with 0.5 cc of human tubal fluid medium without releasing the negative pressure. The sample was placed in the dish and assessed for the presence of sperm under light microscopy (200× magnification).

Testicular Sperm Recovery by Excisional Biopsy

The retrieval of testicular spermatozoa by open excisional biopsy is equivalent to a diagnostic testicular biopsy procedure. To recover testicular spermatozoa, the sampled tissue is disrupted and minced or digested by enzymes to release the spermatozoa from the seminiferous tubules. TESE is even simpler than PESA and may be performed under local anesthesia. In patients with normal spermatogenesis, TESE gives a 100% recovery rate. In patients with testicular failure showing different degrees of maturation arrest, germ-cell aplasia (Sertoli cell-only patterns) recovery rates are approximately 50%. In patients with 47,XXY Klinefelter syndrome, spermatozoa may be recovered from about half of the patients. When spermatozoa are found, ICSI may be performed and pregnancies obtained. Pregnancies have been obtained for patients with 47,XXY Klinefelter syndrome, and the first babies born were reported recently. However, as reported earlier, the fertilization rate after ICSI with spermatozoa obtained from patients with testicular failure is significantly lower. This is confirmed by other studies. Although testicular sperm recovery represents a major breakthrough for patients suffering from non-obstructive azoospermia, the outcome after ICSI remains poor.

In many patients, testicular spermatozoa may be recovered only by excising multiple biopsies,[23, 24] which may cause localized testicular fibrosis.[55] Casuistic reports of less invasive methods by which to retrieve spermatozoa from patients with non-obstructive azoospermia have been published.[18] Although for diagnostic purposes multiple needle aspiration was reported to give reliable results,[43] the technique may have its limitations for recovering testicular spermatozoa in patients with testicular failure. In patients with nonobstructive azoospermia, it may therefore be preferable to perform open biopsies to recover testicular spermatozoa for ICSI or to use thicker aspiration needles.

To minimize tissue damage when taking multiple excisional biopsies, small tissue samples may be taken using a microsurgical approach as proposed by Schlegel. After opening the tunica albuginea, the seminiferous tubules are exposed at 40-80× magnification, and the more distended tubules may be selected for micro-excision. In fact, the technique may be very useful in patients with incomplete Sertoli cell-only syndrome, where there is a substantial difference in diameter for empty and filled tubules. One of the main reasons for

this outcome seems to be histopathological. A general problem with the needle techniques is the difficulty in choosing the right site in the testis. There is evidence of focal areas of normal and abnormal tissue within the testis of participants with non-obstructive azoospermia, and different methods of biopsy require randomized evaluation.[45] Better retrieval results often are reported with open biopsy simply because there is greater potential for more suitable tissue to be obtained. This problem with the needle techniques has been remedied to some extent since practitioners started using a microscope during retrieval procedures.[55, 56] Thorough exploration with a microscope enabled the surgeons to choose the largest tubules in the testis, which are associated with more spermatozoa. Evaluating this newer technique, a study reported better retrieval results with microsurgical sperm extraction compared to conventional open testicular retrieval.[45]

A major advantage of the open approach of freezing testicular tissue and of performing ICSI whenever the final histopathology shows spermatozoa are, the prevention of pointless ovarian stimulation in the female partner. However, in some patients, ICSI may not be proposed because the final histophatology may not show testicular spermatozoa or late spermatids. Although histopathology is the best indicator of whether spermatozoa for ICSI may be present or not,[23-26] in patients with maturation arrest the predictive power is not enough to exclude a patient based on a negative biopsy result.[57] In any case, cryopreservation of testicular spermatozoa may prevent repeat surgery when pregnancy does not occur after ICSI. Overall, considering the low pregnancy rates after ICSI with fresh testicular spermatozoa from patients with testicular failure, the use of frozen-thawed testicular spermatozoa may be preferable to repeat surgery in these patients.

Recently, a simple modified technique was described enabling a faster decision as to the success of TESE, making the switch to donor sperm easier, and, when ovulation induction is performed simultaneously, saving valuable laboratory time and effort in the futile search for testicular tissue spermatozoa when the supernatant is spermatozoa-negative. Basically, the technique consisted of placing the testicular specimen in medium in toto, prior to mechanical shredding. The medium is centrifuged, and the pellet is resuspended and transferred for supernatant sperm detection. Then a wet preparation of the testicular tissue is shredded roughly and inspected for tissue sperm as described.[57,58] The authors found that the supernatant sperm is detected in all specimens where testicular tissue is eventually found, independent of their testicular pathology. When the supernatant was spermatozoa-negative, no spermatozoa were detected in the tissue. For embryos derived from ICSI the fertilization rate of the supernatant sperm is significantly higher than testicular sperm from the tissue (52% vs. 44%), whereas the pregnancy rate is comparable.

Other Surgical Methods of Spermatozoa Retrieval

Reports have been published describing alternative methods for recovering spermatozoa from the male genital tract, e.g. from the vas deferens for conventional IVF. These techniques are reported to yield many spermatozoa and may be especially useful in patients with anejaculation in whom vibrostimulation or electroejaculation has failed. However, both vasal sperm retrieval and retrograde epididymal sperm collection may lead to the recovery of more dysfunctional spermatozoa or spermatozoa with DNA damage, since it is known that, in patients with obstruction, the most distal spermatozoa are the most senescent.[58-62]

Implications for Practice

Data from randomized trials are insufficient to recommend any particular surgical sperm retrieval techniques for either obstructive or non-obstructive azoospermia. Non-obstructive azoospermia is a difficult area to analyze as the physiology of the testis may vary between individuals. Techniques are modified rapidly and considerable variation exists among different centers and surgeons. Using the least invasive and simplest (and thereby most cost-effective) method for surgical retrieval of sperm is a logical approach in the absence of evidence to support more invasive or more technically difficult methods. The more invasive methods should currently be reserved for situations where sperm cannot be retrieved by a less invasive technique (such as needle aspiration of the epididymis or testis) or for evaluation in the context of a randomized trial.

Implications for Research

The onus remains with those in support of more invasive techniques of surgical retrieval of sperm that require greater surgical expertise to demonstrate that these techniques can be justified by performing suitably powered

randomized clinical trials. Such trials need to have a particular focus upon:

1. Clear definition of the population of men studied in terms of etiology of azoospermia;
2. Use of clinically relevant outcomes: not only clinical pregnancy and live birth rates, but also the rate of birth of a normal healthy baby, and certainly not simply the success of retrieval of sperm suitable for ICSI;
3. Cost-effectiveness (with inclusion of a cost-benefit analysis).

As the prevalence of azoospermia is low, it remains unlikely that a single unit will attain numbers to confer sufficient power to such a trial. Large multicenter trials would increase the power and confer generalizability to the results.

Latest Update Study

An updated Cochrane review was published very recently (2008) by Drs. Van Peperstraten, Proctor, Johnson and Philipson about the techniques for surgical retrieval of sperm prior to intracytoplasmic sperm injection for azoospermia.[63] The authors concluded that evidence is still insufficient to recommend any specific sperm retrieval technique for azoospermic men undergoing ICSI. In the absence of evidence to support more invasive or more technically difficult methods, use of the least invasive and simplest technique available is recommended. Further randomized trials are warranted, preferably multicenter trials. The classification of azoospermia as obstructive and non-obstructive appears to be relevant to a successful clinical outcome, and a distinction according to the cause of azoospermia is important for future clinical trials.

Summary

Since the introduction of ICSI, both epididymal and testicular spermatozoa have been used successfully to obtain pregnancies. Different methods for recovering epididymal or testicular spermatozoa have been described, and each has its drawbacks and advantages. The question may therefore arise as to which method is preferable. From data in the literature, it appears that percutaneous aspiration of the testis may be the method of choice in patients with normal spermatogenesis, since the sperm recovery rate is high and patients experience less pain than those undergoing an open biopsy. In patients with obstructive azoospermia who have not had a work-up or have had an incomplete one, MESA is the preferred method because a full scrotal exploration can be performed and, whenever indicated, a vasoepididimostomy may be performed concomitantly.

In azoospermic patients showing different degrees of maturation arrest or germ cell aplasia, recovery rates are approximately 50% after multiple open biopsies. Because the fertilization rates after ICSI are significantly lower and because of high fertilization rates, ongoing pregnancies are obtained in only 10% of ICSI cycles. The surgical recovery of testicular spermatozoa by multiple open biopsies with cryopreservation, followed by thawing only after histopathology demonstrates the presence of spermatozoa may be the method of choice by which to prevent repeat surgery and pointless ovarian stimulation in the female partner.

REFERENCES

1. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992;340:17-18.
2. Palermo GD, Schlegel PN, Hariprashad JJ, Ergün B, Mielnik A, Zaninovic N, Veeck LL, Rosenwaks Z. Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. Hum Reprod 1999; 14:741-48.
3. Pasqualotto FF, Rossi LM, Guilherme P, Ortiz V, Iaconelli Jr A., Borges Jr. Etiology-specific outcomes of intracytoplasmic sperm injection in azoospermic patients. Fertil Steril 2005;83:606-11.
4. Pasqualotto FF, Rossi-Ferragut LM, Rocha CC, Iaconelli A Jr., Borges E Jr. Outcome of in vitro fertilization and intracytoplasmic injection of epididymal and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. J Urol 2002; 167: 1753-56.
5. Pasqualotto FF, Rossi-Ferragut LM, Rocha CC, Iaconelli A, Ortiz V, Borges E. The efficacy of repeat percutaneous epididymal sperm aspiration procedures. J Urol 2002; 169: 1779-81.
6. Borges E Jr., Rossi-Ferragut LM, Pasqualotto FF, Rocha CC, Iaconelli A. Is there a difference between different intervals after vasectomy and the reproductive capacity from vasectomyzed men? J Assist Reprod Gen 2003; 20(1): 33-37.
7. De Cassia Sávio Figueira R, Madaschi C, Nichi M, Rodrigues D, Pasqualotto FF, Iaconelli A Jr, Borges E Jr. A comparison of post-thaw results between embryos arising from intracytoplasmic sperm injection using surgically retrieved or ejaculated spermatozoa. In press, Fertil Steril 2008.
8. Irvine DS. Epidemiology and aetiology of male infertility. Hum Reprod 1998; 13(Suppl 1):33-44.

9. Borges E Jr, Rossi-Ferragut LM, Pasqualotto FF, Santos DR, Rocha C, Aoki T, Iaconelli A, Jr. Testicular sperm results in elevated miscarriage rates compared to epididymal sperm in azoospermic patients. São Paulo Med J 2002;120(4): 122-26.
10. Pasqualotto FF, Locambo CV, Athayde KS, Arap S. Measuring male infertility: epidemiological aspects. Rev Hosp Clin Fac Med S Paulo 2003;58(3): 173-78.
11. Pasqualotto FF, Lucon AM, Sobreiro BP, Pasqualotto EB, Arap S. Effects of medical therapy, alcohol, smoking and endocrine disruptors on male infertility. Rev Hosp Clin Fac Med S Paulo 2004;59(6): 375-82.
12. Pasqualotto FF, Agarwal A, Srivastava M, Thomas, AJ, Jr.: Fertility outcome after repeat-vasoepididymostomy. J Urol 1999; 162: 1626-28.
13. Belker AM, Thomas AJ Jr, Fuchs EF, Konnak JW, Sharlip ID. Results of 1,469 microsurgical vasectomy reversals by the Vasovasostomy Study Group. J Urol 1991; 145: 505-11.
14. Jarow JP, Budin R E, Dym M, Zirkin BR, Noren S, Marshall FF. Quantitative pathologic changes in the human testis after vasectomy. A controlled study. NEJM 1985; 313:1252-56.
15. Jarow JP, Oates RD, Buch JP, Shaban SF, Sigman M. Effect of level of anastomosis and quality of intraepididymal sperm on the outcome of end-to-side epididymovasostomy. Urol 1997; 49(4):590-95.
16. Tesarik J, Mendoza C, Testart J. Viable embryos from injection of round spermatids into oocytes. NEJM 1995;333: 525.
17. Antinori S, Versaci C, Dani G. Fertilization with human testicular spermatids: four successful pregnancies. Hum Reprod 1997;12; 286-91.
18. Lewin A, Reubinoff B, Porat-Katz A, Weiss D, Eisenberg V, Arbel R, Bar-el H, Safran A. Testicular fine needle aspiration: the alternative method for sperm retrieval in non-obstructive azoospermia. Hum Reprod 1999; 14(7):1785-90.
19. The Male Infertility Best Practice Policy Committee of the American Urological Association and the Practice Committee of the American Society for Reproductive Medicine, American Urological Association, Baltimore, Maryland; and American Society for Reproductive Medicine, Birmingham, Alabama. Report on evaluation of the azoospermic male. Fertil Steril 2006; 86(Suppl.4), S210.
20. Sheynkin Y, Zhen Y, Menendez S, Liotta D, Veeck L, Schlegel P. Controlled comparison of percutaneous and microsurgical sperm retrieval in men with obstructive azoospermia. Hum Reprod 1998; 13(11):3086-89.
21. Van Rumste MME, Evers JLH, Farquhar CM, Blake DA. Intra-cytoplasmic sperm injection versus partial zona dissection, subzonal insemination and conventional techniques for oocyte insemination during in-vitro fertilisation (Cochrane Review). In: The Cochrane Library, 4, 2000. Oxford: Update Software.
22. Sharif K. Reclassification of azoospermia: the time has come? Hum Reprod 2000; 15(2):237-38.
23. Tournaye H. Surgical sperm recovery for intracytoplasmic sperm injection: which method is to be preferred? Hum Reprod 1999; 14: 71-81.
24. Tournaye H, Casen K, Aytoz A, Nagy Z, Van Steirteghem A, Devroey P. Fine needle aspiration versus open biopsy for testicular sperm recovery: a controlled study in azoospermic patients with normal spermatogenesis. Hum Reprod 1998; 13: 901-04.
25. Tournaye H, Liu J, Nagy Z. Correlation between testicular histology and outcome after intracytoplasmic sperm injection using testicular sperm. Hum Reprod 1996; 11: 127-32.
26. Tournaye H, Goossens A, Ubaldi F. Are there any predictive factors for successful testicular sperm recovery? Hum Reprod 1997;12: 80-86.
27. Tournaye H, Clasen K, Aytoz A. Fine needle aspiration versus open biopsy for testicular sperm recovery: a controlled study in azoospermic patients with normal spermatogenesis. Hum Reprod 1998; 13, 901-04.
28. Oates RD, Lobel SM, Harris DH. Efficacy of intracytoplasmic sperm injection using intentionally cryopreserved epididymal spermatozoa. Hum Reprod 1996;11:133-38.
29. Craft I, Bennett V, Nocholson N. Fertilising ability of testicular spermatozoa. Lancet 1993;342: 864.
30. Craft I, Tsirigotis M, Courtauld E, Farrer-Brown G. Testicular needle aspiration as an alternative to biopsy for the assessment of spermatogenesis. Hum Reprod 1997;12: 1483-87.
31. Craft I, Tsirigotis M, Bennett V. Percutaneous epididymal sperm aspiration and intracytoplasmic sperm injection in the management of infertility due to obstructive azoospermia. Fertil Steril 1995;63:1038-42.
32. Tsirigotis M, Pelekanos M, Yazdani N. Simplified sperm retrieval and intracytoplasmic sperm injection in patients with azoospermia. Br J Urol 1995;76:765-68.
33. Collins GN, Critchlow JD, lau MWM, Payne SR. Open versus closed epididymal sperm retrieval in men with secondary obstructed vasal systems – a preliminary report. Br J Urol 1996;78: 437-39.
34. Devroey P, Liu J, Nagy Z. Ongoing pregnancies and birth after intracytoplasmic sperm injection with frozen-thawed epididymal spermatozoa. Hum Reprod 1995;10: 903-06.
35. Nicopoullos JDM, Gillin-Smith C, Almeida PA, Norman-Taylor J, Grace I, Ramsay JWA. Use of surgical sperm retrieval in azoospermic men: a meta-analysis. Fertil Steril 2004; 82:691-700.
36. Girardi SK, Schlegel P. MESA: review of techniques, preoperative considerations and results. J Androl 1996;17:5-9.
37. Yamamoto M, Hibi H, Miyake K, Asada Y, Suganuma N, Tomoda Y. Microsurgical epididymal sperm aspiration versus epididymal micropuncture with perivascular nerve stimulation for intracytoplasmic sperm injection to treat unreconstructable obstructive azoospermia. Archives of Andrology 1996;;36:217-24.

38. Carpi A, Fabris FGM, Palego P, Coscio G, Romani R, Nardini V, Rossi G. Fine-needle and large-needle percutaneous aspiration biopsy of testicles in men with nonobstructive azoospermia: safety and diagnostic performance. Fert Steril 2005;83:1029-33.
39. De Croo I, Van der Elst J, Everaert K, De Sottos P, Dhont M. Fertility, pregnancy and embryo implantation rates after ICSI in cases of obstructive and non-obstructive azoospermia. Hum Reprod 2000; 15: 1383-88.
40. Friedler S, Raziel A, Strassburger D, Schachter M, Soffer Y, Ron-El R. Factors influencing the outcome of ICSI in patients with obstructive and non-obstructive azoospermia: a comparative study. Hum Reprod 2002; 17: 3144-21.
41. Mercan R, Urman B, Alatas C, Aksoy S, Nohoglu A, Isiklar A, Balaban B. Outcome of testicular sperm retrieval procedures in non-obstructive azoospermia: percutaneous aspiration versus open biopsy. Hum Reprod 2000; 15: 1548-51.
42. Belker AM, Sherins RJ, Dennison-Lagos L, Thorsell LP, Schulman JD. Percutaneous testicular sperm aspiration: a convenient and effective office procedure to retrieve sperm for in vitro fertilization with intracytoplasmic sperm injection. J Urol 1998; 160: 2058-62.
43. Turek PJ, Cha I, Ljung B-M. Systematic fine-needle aspiration of the testis: correlation to biopsy and results of organ "mapping" for mature sperm in azoospermic men. Urology 1997; 49: 743-48.
44. Schulze W, Thoms F, Knuth UA. Testicular sperm extraction: comprehensive analysis with simultaneously performed histology in 1418 biopsies from 766 subfertile men. Hum Reprod 1999; 14: 82-96.
45. Amer M, Haggar S, Moustafa T, El-Naser T, Zohdy W. Testicular sperm extraction: impact of testicular histology on outcome, number of biopsies to be performed and optimal time for repetition. Hum Reprod 1999; 14 (12):3030-34.
46. Amer A, Ateyah A, Hany R, Zohdy W. Prospective comparative study between microsurgical and conventional testicular sperm extraction in non-obstructive azoospermia: follow-up by serial ultrasound examinations. Hum Reprod 2000; 15(3):653-56.
47. Ezeh U, Moore H, Cooke I. A prospective study of multiple needle biopsies versus a single open biopsy for testicular sperm extraction in men with non-obstructive azoospermia. Hum Reprod 1998; 13(11):3075-80.
48. Friedler S, Raziel A, Strassburger D, Soffer Y, Komarovsky D, Ron-El R. Testicular sperm retrieval by percutaneous fine needle sperm aspiration compared with testicular sperm extraction by open biopsy in men with non-obstructive azoospermia. Hum Reprod 1997; 12(7):1488-93.
49. Jaroudi K, Coskun S, Hollanders J, Al-Hassan S, Al-Sufayan H, Atared A, Merdad T. Advanced surgical sperm recovery is a viable option for intracytoplasmic sperm injection in patients with obstructive or nonobstructive azoospermia. Fertil & Steril 1999; 72(3):479-83.
50. Rosenlund B, Kvist U, Plöen L, Lundh Rozell B, Sjöblom P, Hillensjö T. A comparison between open and percutaneous needle biopsies in men with azoospermia. Hum Reprod 1998; 13(5):1266-71.
51. Segal S, Zohav E, Katz N, Kinchin B, Popescu M, Gemer O, Sassoon E. The use of an automatic biopsy system for the retrieval of testicular spermatozoa for intracytoplasmic sperm injection. Hum Reprod Abstracts of the 11th Annual meeting of ESHRE, Book 2; 10:101, 1995.
52. Hovatta O, Moilanen J, Von Smitten K, Reima I. Testicular needle biopsy, open biopsy, epididymal aspiration and intracytoplasmic sperm injection in obstructive azoospermia. Hum Reprod 1995; 10:2595-99.
53. Mallidis C, Baker GHW. Fine needle tissue aspiration of the testis. Fertil Steril 1994; 61:367-75.
54. Morey AF, Deschon GE Jr, Rozanski TA, Dresner ML. Technique of biopsy gun testis needle biopsy. Urol 1993;42:325-26.
55. Schlegel P. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. Hum Reprod 1999; 14(1):131-35.
56. Schlegel P, Su L. Physiological consequences of testicular sperm extraction. Hum Reprod 1997; 12(8):1688-92.
57. Mulhall JP, Burgess CM, Cunningham D. Presence of mature sperm in testicular parenchyma of men with nonobstructive azoospermia: prevalence and predictive factors. Urol 1997;49: 91-96.
58. Haimov-Kochman R, Imbar T, Lossos F, Nefesh I, Zentner B-S, Moz Y, Diana P, Bdolah Y, Hurwitz A. Technical modification of testicular sperm extraction expediates testicular sperm retrieval. In press, Fertil Steril, 2008.
59. Silber SJ, Nagy Z, Liu J, Tournaye H, Lissens W, Ferec C, Liebaers I, Devroey P, Van Steirteghem AC. The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility. Hum Reprod 1995; 10(8):2031-43.
60. Jansen R. Overcoming infertility. New York: WH Freeman, 1997.
61. Hirsch A, Mills C, Tan SL. Pregnancy using spermatozoa aspirated from the vas deferens in a patient with ejaculatory failure due to spinal injury. Hum Reprod 1993;8: 89-90.
62. Utsunomiya T, Ando K, Ogata S. Successful pregnancies using new sperm collection method with retrograde epididymal sperm aspiration (RESA) and micro-fertilization on severe male infertility. Jpn Fertil Steril 1997;42:39-42.
63. Van Van Peperstraten A, Proctor ML, Johnson NP, Philipson G. Techniques for surgical retrieval of sperm prior to intra-cytoplasmic sperm injection (ICSI) for azoospermia. Cochrane Database of Systematic Reviews 2008, Issue 2. Art. No.: CD002807. DOI: 10.1002/14651858.CD002807.pub3.

CHAPTER 21

Human Sperm Cryopreservation

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ABSTRACT

Human sperm cryopreservation has become widely accepted in our society. It has been proven to be a very successful method to keep the hope of a family alive for many men.

In early 1940s cattle breeders started cryopreserving bull semen. The cryopreservation of human semen started in 1950s with more refined techniques designed for human sperm.

Since then continuous ongoing research has developed more sophisticated techniques for human semen cryopreservation. This chapter is an introduction to the human sperm cryopreservation, the latest techniques of the sperm cryopreservation and its significance in assisted reproductive techniques.

SPERM CRYOPRESERVATION: HISTORY

Efforts to maintain the fertilizing capacity of mammalian spermatozoa for extended times have been made for at least 135 years. Having observed survival of human spermatozoa that had been cooled to –150°C in 1866, an Italian physician P. Mantegazza proposed the concept of a human sperm bank to store semen specimens.[1] Since the birth of Louise Brown, the first test tube baby in the world on 25th July, 1978 by the pioneering work of Dr Robert Edwards and Dr Patrick Steptoe, it has been reported that three million babies have been born by assisted reproductive technology (ART). Human sperm cryopreservation has become a routine procedure since then in any assisted reproductive technology laboratories.

SPERM CRYOPRESERVATION: SIGNIFICANCE

Cryopreservation is widely used in many assisted reproductive programs to preserve male fertility, for example, before cytotoxic chemotherapy,[2] radiotherapy, or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction. Freezing of sperm before initiation of treatment provides patients "fertility insurance" and may allow them to father their own children through the use of intrauterine Insemination or *in vitro* Fertilization techniques such as intra cytoplasmic sperm injection (ICSI).

Cryopreservation of sperm is mandatory in donor-insemination programs. The use of frozen semen allows screening of donors for infectious diseases such as HIV and hepatitis B before release for insemination.[3-6] Cryopreservation is also widely used for storage of sperm retrieved from azoospermic patients who have undergone testicular sperm extraction or percutaneous epididymal sperm aspiration (PESA), avoiding the need of repeated biopsies or aspiration.

SPERM CRYOPRESERVATION: PURPOSE

At present, the most commonly used cryofreezing procedures are in propylene straws/vials of different sizes and volumes and it has been documented by several researchers.[7-9]

Several studies have aimed to find correlations between different parameters of frozen-thawed semen and fertility in several species including stallions.

Unfortunately, so far, no single *in vitro* parameter has been identified to predict fertility. Sperm motility is the most commonly used criterion for evaluating quality of cryopreserved semen. It has been found that Computer Assisted Sperm Analysis (CASA) is sufficient for clinical use in crucial individual motility patterns.[10-12] Nevertheless it may be inadequate for determining an individual's fertility as only motility evaluation does not predict the fertility potential of the sperm cells. Different probes have been used to measure sperm viability and acrosome reaction in different species.[13-15]

It has been argued that such patients undergoing procedures that may lead to testicular failure or ejaculatory dysfunction should be offered cryopreservation of semen.[16] Although in 1980 Braken and Smith reported that only 4% of patients had semen of suitable quality for a fertility program,[17] the advent of ICSI overcame these limitations, it allows even low quality samples to be used successfully.[18]

Regarding the safety of the cryopreservation techniques, questions about the possibility of cross contamination through the liquid nitrogen could be raised. Hepatitis C virus has a low rate of sexual and intra-familial transmission,[19] and till date there is not a single case report where it has been found to be transmitted by stored bone marrow or semen. Some studies of semen, saliva, and vaginal secretions in HCV-positive patients have failed to find HCV RNA.[20,21] It is believed that semen samples may have very small viral load that would not survive even the liquefaction time. HCV is a RNA virus and only have RNA dependent RNA polymerase to reverse transcript to RNA strand only, which are not at all stable and can not be integrated to host DNA. A case of DNA virus hepatitis B acquired from stored bone marrow was recently described[22] and hence awareness is required for all potentially transmissible agents. With isolated storage facilities now provided, all patients who are positive for antibody to hepatitis C virus should be able to preserve their long term fertility.

Within the many reasons for sperm cryopreservation, this technique could assist the male partner in an IVF couple in the following situations: concern about the stress of the collection of semen on demand, any history of poor specimens collected for IVF, traveling husband (not available at the particular time of the insemination) or in case of oligozoospermic samples, when collection of more than one ejaculate are pooled for IUI/IVF. Spermatozoa cryopre-servation is also of interest for maintaining genetic diversity in endangered species, especially those in zoological parks which are candidate species for captive breeding programs.[23]

CRYOPROTECTANTS: INDICATIONS

In 1938, the survival of human spermatozoa stored at the temperature of solid carbon dioxide was reported. Despite this finding, it was not possible to start using cryopreserved semen in breeding programs until a considerable observation taken up by Parkes in 1945.[24] The single most important development came with the significant discovery by Polge, Smith, and Parkes in 1949[25] that glycerol could act as a cryoprotectant for spermatozoa. The initial experiments with glycerol were performed using fowl spermatozoa but were quickly followed by the successful preservation of bull spermatozoa[26] with the first calf from Artificial Insemination using frozen/thawed spermatozoa reported in 195l.[27]

The use of glycerol had allowed bull spermatozoa to survive at the comparatively slow freezing rates obtained using methanol cooled with solid CO_2, and with semen contained in large glass-freezing ampoules. Effective cryoprotectants are preferentially excluded from proteins; thereby it is more stabilized and avoids unfolding or denaturation.[28]

Such cryoprotectants are hydrophobic enough to pass cell membranes easily and would be hydrophilic enough to readily mix with water. If the cryoprotectants are toxic then it may act more by hydrogen-bonding with water than by colligative interference with ice formation. It implies that understanding the molecular mechanisms of the toxicity of cryoprotective agents would be an important advance step toward finding means of reducing toxicity. Cryoprotectant toxicity and destabilization of polypeptides in cell membranes have been documented.[29]

In general, cryoprotectant toxicities are lower at lower temperature, and may even become negligible if the cryoprotectants can be introduced at a low enough temperature. The challenges that cells have to overcome to survive after freeze-thaw are not their ability to endure storage at low temperature; rather, it is the lethality of an intermediate zone of temperature (– 15 to – 60°C) that cells must pass through during cooling as well as during warming.[30] As the cells approach the freezing temperature, the water outside the cell freezes first,

removing liquid solvent from the extracellular broth and resulting in increased intracellular osmolarity.

There are two groups of cryoprotectants used in routine cryogenic work. One is cell membrane permeable and another one is membrane non-permeable. Cell membrane permeable cryoprotectants viz. glycerol, dimethyl sulfoxide (DMSO) and membrane non-permeable cryoprotectants viz. polyhydroxyethyl starch, glycine are being used randomly. At the present time different sugar molecules like glucose, sucrose, raffinose, etc. have also been frequently used as cryoprotectant in routine work.[31] Most of these cryoprotectants are used at a concentration of 5 to 10%.[32]

Among these, glycerol is the most commonly used cryoprotectant for mammalian as well as human spermatozoa.[33, 34] Conversely, Jeyendran[35] showed that spermatozoa might become dependent on glycerol so that once it is removed, motility rapidly decreases. For the better recovery of the motile cells along with the cryoprotectants several other natural extenders viz. egg yolk, or synthetic extenders viz. Zwitterion buffers containing TRIS [hydroxy-methylaminomethane], TES [N-TRIS (hydroxymethyl) methyleaminoethanesulfonic acid], or combination of these are generally being used routinely.[36, 37] Sodium citrate, another substance added to extending buffers, may give better result.[38, 39]

Nowadays HSPM (Human Sperm Preservation Medium) has become very popular in many laboratories.[40, 41] It has been reported that TYB over HSPM gives better result in motility recovery both containing 7% (v/v) glycerol.[42] But in a comparative study, TYB in contrast with HSPM can be recommended as a first choice cryoprotectant for semen preservation in order to avoid extra chromatin structure damage and morphology alterations of spermatozoa not only for infertile subjects pursuing assisted reproduction, but also for donor samples.[43]

The importance of sperm morphology in the assessment of male fertility is evident in a large number of studies.[44-46] Many authors consider it to be the best semen parameter in predicting fertilization potential,[47-49] pregnancy outcome in intrauterine insemination[50, 51] as well as fertilization and pregnancy rate in programs of *in vitro* fertilization (IVF).[52-54] Hence sperm morphology should be evaluated before and after freezing prior to introduction of any cryopreservation procedure in the lab.

EVALUATION OF SPERM POST-CRYOPRESERVATION

Chromatin condensation is now accepted as one of the most important of the numerous sperm function tests.[55] Many researchers believe that the presence of abnormal sperm chromatin may also lead to reproductive failure or to habitual abortion following fertilization.[56] As egg yolk and serum albumin both diluents are of animal origin, to reduce the risk of contamination, the cryoprotective property of phospholipids extracted from lecithin was evaluated and found to be effective when supplemented with dimethyl sulfoxide and glycerol.[57]

Ollero et al,[58] showed no improvement of the cooling-induced cell surface damage by freezing in the presence of bovine seminal plasma, proline, glycine-betaine (found in Antarctic bacteria as their anti-freezing phospholipids) and phosphatidylcholine. In that comparative study, vitamin E and cholesterol collectively showed a significant good result with that cryoprotectant. Even, the best protective effects were found by employing seroalbumin and lactalbumin. In addition it was been observed that, freezing in the presence of bovine lactalbumin, resulted in a significantly better maintenance of the cellular viability and of the centrifugal counter-current distribution (CCCD) heterogeneity in respect to fresh cells.

Ollero et al,[59] tested the cryoprotective ability of four different extenders [Triladyl-yolk (20% Tridyl, 20% egg-yolk) Salamon (9.9% raffinose, 2% sodium citrate, 15% egg-yolk, 5% glycerol), INIA (6.31% TES, 1.51% TRIS, 6% egg-yolk, 3% glycerol), and Fiser (F1: 3.25% TRIS, 9.3% fructose, 1.7% citric acid, 25% egg-yolk, 2% glycerol, F2: 0.68% sodium citrate, 0.15% TES, 0.36% glycine, 10.18% lactose, 1.18% raffinose, 0.5% fructose, 3.95% dextran); 12% of the obtained solution was replaced by the same volume of glycerol].

There was a dramatic loss of heterogeneity, viability and motility during freezing thawing of ram spermatozoa were observed that it could be prevented at least partially when Fiser's extender containing egg-yolk and glycerol was used for preservation among the four different extenders with bovine lactalbumin and serum albumin, vitamin E, dithiothreitol, bull seminal plasma, glycine betaine, proline, phosphatidylcholine and cholesterol. The antioxidant properties of Trehalose based hypertonic extenders shows effectiveness in membrane Cryopreservation.[60] Even Yamashiro et al,[61] supported

this cryoprotective fact of trehalose, which acts as a effective freezability increasing extender with egg-yolk, in a mammalian animal model.

Freezing Procedures

Neat (unprocessed) Semen

1. Sperm cyroprotectant II is added drop-wise to unprocessed semen ensuring thorough mixing after adding each drop, dilution 1:4 (e.g 0.1 mL SCPII to 0.4 mL semen).
2. Fill vials with sperm suspension and equilibrate them in freezer – 20°C for 8 minutes.
3. Straws are then placed vertically 14 to 17 cm above the liquid nitrogen surface and left for 2 hours for the vapor phase.
4. Vials are then transferred quickly into the liquid nitrogen and stored in the liquid nitrogen.

Prepared Semen (Pre-freeze Preparation)

1. The ejaculate is prepared on a density gradient and washed with a sperm wash media.
2. Sperm cyroprotectant II is added drop-wise to the processed semen ensuring thorough mixing after adding each drop, dilution 1:4 (e.g 0.1 mL SCPII to 0.4 mL semen)
3. Fill vials with sperm suspension and equilibrate them in freezer – 20°C for 8 minutes.
4. Straws are then placed vertically 14 to 17 cm above the liquid nitrogen surface and left for 2 hours for the vapor phase.
5. Vials are then transferred quickly into the liquid nitrogen and stored in the liquid nitrogen.

SPECIAL CRYOPRESERVATION CASES

Oligozoospermic Specimen

The state of 'virtual' azoospermia, is defined as the occasional presence of few sperm in the ejaculate, and is distinguished from 'absolute' azoospermia, defined as the lack of sperm in the ejaculate.[62] Therefore with the existence of such states, meticulous search and cryopreservation can rescue IVF-ICSI cycles and prevent TESA procedures, donor sperm usage, or cycle cancellation in a select group of patients.[63] For oligozoospermic man pooling of previously banked semen specimen in male who is diagnosed oligozoospermia and using this pooled and prepared sample, for one insemination could increase the chance of pregnancy. However, the dilemma encountered with the practice of pooling cryopreservation is at what frequency and intervals to perform the sperm analysis for the purpose of eventual cryopreservation.[63,64]

Testicular Sperm Aspiration (TESA)/Percutaneous Epididymal Aspiration (PESA)

Testicular tissue from prepubertal boys does not contain mature spermatozoa and so cannot be used for assisted reproductive techniques without maturation *in vitro*. As it is not possible to collect an ejaculated sample, cryopreservation of surgically retrieved testicular tissue may be the only option.[65, 66] However, efficient cryopreservation is somewhat more difficult for testicular tissue than for semen because of low concentration and motility of spermatozoa. Nevertheless, only a few spermatozoa surviving cryopreservation is needed for ICSI. In a study, it is recorded that by mincing the testicular biopsy before freezing, the quality of testicular spermatozoa is preserved more efficiently.[67,68] Besides the practical difficulties yet to be overcome in the use of immature sperm, retrieval of such tissue presents legal and ethical dilemmas because the child is unlikely to be old enough to give informed consent.

Sperm Banking

Nowadays infertility has become a major issue and due to many infertility programs voluntary sperm donation is highly appreciated over replacement donor, as many transient or occult sexually transmitted infections may not be properly detected and gives a birth of unhealthy child. It has been recorded that occult infection with HIV does occur in a greater frequency than do progressive infection.[69] A Sperm Bank is a facility that collects and cryopreserves known healthy human donor's sperm sample primarily for the purposes of artificial insemination. Using anonymous or known healthy donor sperm from a sperm bank is a safe alternative. After collection of the sperm by manual stimulation, the sample is extensively analyzed to ensure the quality and pathogenicity of the sperm sample. Even donors are frequently tested and monitored and all sperm is cryogenically stored a minimum period of a few months, without using in any infertility program, to make sure that the donor as well as the collected and stored sperm is healthy and normozoospermic.[70, 71]

Traveling Husband

Sperm cryopreservation can be also used for timing issues (urgent travel during ART cycles), where achieving pregnancy to get their own genetic child, is the prime target of the infertility program, whenever donor sperm are not accepted by the couples.[72] Ovulation time variability; as the shortest menstrual cycle minus 18 days is the 1st day of fertile period and the longest menstrual cycle minus 10 days is last day of fertile period, so the 8th to 21st day of each cycle counting from first day of menstrual period is considered as fertile period. Furthermore if female factor infertility has developed a blockage on her single fallopian tube (can be determined by tubal patency test) pregnancy is possible with only one fallopian tube as long as the female subject have one or both ovulating ovaries. Normally, a single egg is released each month by one of the ovaries and travels down through fallopian tube, where a sperm may fertilize it after an unprotected natural sexual intercourse or assisted reproductive technologies (ART).[73]

A strong primary sex drive and regular sexual intercourse surely help to attain a pregnancy. If in that case the husband has to travel around, due to his job or business, or lives elsewhere he may miss the exact fertile period of his own partner, who is having one-sided blockage on fallopian tube. It may happen that Husbands and wife's schedule does not synchronize at the time of the treatment schedule between wife's fertile period, wife can continue their future reproductive plan and can conceive a child by thawing her partner's precryopreserved sperm by having an intrauterine insemination (IUI). Even some of the government regulations allow the removal of sperm from the husband's dead body at the request of his wife or common law wife.[74]

Occupational Risk

Those male subjects, who are exposed to hazardous materials, toxicants or radiation due to their job link, can also store their sperm for future family planning. Men in defense or security may have to face life-threatening situations and can avail cryopreservation before going to battlefield. Professional sportsperson or heavy weight lifter, though use protective gear, are more prone to accidental serious testicular injury like **testicular torsion** and **testicular rupture**, can also preserve their sperm for their future reproductive planning. However, some ethical issues may be needed for husband postmortem sperm retrieval.[75, 76]

Cancer Patients (Hematological, Testicular or Other Malignancy)

Successful cryopreservation of sperm would benefit men recently diagnosed with cancer who want to retain their fertility potential.[77] Møller et al,[76] established the connection between male subfertility and subsequent testicular cancer. Lass et al[77] and Meirow et al,[78] showed that men with malignant disease like contralateral testis in men with testicular cancer, lymphoma, leukemia, sarcoma, carcinoma, have poor sperm quality at the time of diagnosis of their illness.

Further deterioration occurs due to the damaging effect of chemotherapeutic toxic agents on spermatogenesis process, which may be temporary or permanent.[79,80] From the report by Agarwal et al[79] 20 years semen banking experience revealed some critical facts that pretreatment semen quality (pre-freeze and post-thaw) in patients with cancer is poorer compared with healthy donors. Nevertheless the percentage decline in semen quality (from pre-freeze to post-thaw) in subjects with cancer is similar to normal donors. This implies that the effect of cryodamage on spermatozoa is the same on subjects with or without cancer. Even the stage of cancer in patients with testicular cancer or Hodgkin's disease shows no significant relationship to their semen quality.

The majority of subjects with malignancy have sufficient suitable sperm for freezing before starting chemotherapy. Endurance of men aged between 15 years to 44 years with cancer has improved to 91% in recent years.[82] Hallak et al suggested that, after checking sperm quality in 25 male subjects with leukemia before and after cryopreservation including 3 male subjects with acute myeloid leukemia (AML), sperm cryopreservation should be offered to all male subjects of reproductive age, independent of the stage of their disease, before the initiation of therapy for malignancies.[81,82] Progress in assisted reproductive techniques can also secure the fertility potential of these male subjects by the advanced micromanipulation specifically the intra cytoplasmic sperm injection. Cryopreservation is safe and inexpensive, and gives patients a chance, particularly those who have not completed their family yet, to establish pregnancies in the future with an assisted reproductive technique.

Lass et al[77] showed that their possibility of fathering their own genetic children is quite high. Furthermore, subject understands that their fertility potential is secured and preserved, which in-turn would help the subject to fight in the emotional battle against the cancer.[83, 84]

Future Perspectives in Sperm Cryobiology

Molecular characterization can be occur in host genetic factors, influencing post-thaw semen quality, are in need to identify the molecular markers, as they show association with semen freezability quality. Whether any genetic damage (i.e. DNA strand breakage or mutation) is also induced by cryopreservation is still unclear. However, previous data has indicated that this is likely. Kopeika et al[83] study also indicates that cryopreservation of sperm may affects the future development of the embryos and might induce genetic damage of sperm that can not be corrected by the DNA repair system of the egg. Even after such a long period of trial and error a standardized and effective, referring to cryopreservation injury, protocol for cryopreservation is yet to attain.[84]

More over increasing the awareness of general practitioners, oncologists, hematologists, and patients synchronizing the knowledge to the new opportunities and importance of cryopreservation in assisted reproductive technology opened to them, so far, in infertility management should be in high priority.

REFERENCES

1. Bunge RG, Keettel WC, Sherman JK. Clinical use of frozen semen: report of four cases. Fertil Steril 1954;5:520-29.
2. Sanger WG, Olson JH, Sherman JK. Semen cryobanking for men with cancer—criteria change. Fertil Steril 1992;58:1024-27.
3. Smith AU, Polge C. Survival of spermatozoa at low temperatures. Nature 1950;166(4225):668-69.
4. Stewart D. Storage of bull spermatozoa at low temperatures. Vet. Record 1951;63:65-66
5. Watson PF. The Preservation of semen in mammals, In: Oxford Reviews of Reproductive Biology, (Finn, CA, ed.), Oxford University Press, Oxford, UK, 1979;283-350.
6. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949;164(4172):666.
7. Martin JC, Klug E, Guntzel AR. Centrifugation of stallion semen and its storage in large volume straws. J Reprod Fertil Suppl 1979;(27):47-51.
8. Tischner M. Evaluation of deep-frozen semen in stallions. J Reprod Fertil Suppl 1979;(27):53-59.
9. Palmer E. Factors affecting stallion semen survival and fertility. Proc. 10th Cong Anim AI 1984;3:377-79.
10. Amann RP, Pickett BW. Principles of cryopreservation and review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987;7: 145-73.
11. Jasko DJ, Little TV, Smith K, Lein DH, Foote RH. Objective analysis of stallion sperm motility. Theriogenology 1988;30(6):1159-67.
12. Blach EL, Amann RP, Bowen RA, Frantz D. Changes in quality of stallion spermatozoa during cryopreservation: plasma membrane integrity and motion characteristics. Theriogenology 1989;31(2):283-98.
13. Graham JK, Kunze E, Hammerstedt RH. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. Biol Reprod 1990;43(1):55-64.
14. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR- 14 and propidium iodide. Biol Reprod 1995;53(2):276-84.
15. Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP, et al. Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenology 1997;48(2):299-312.
16. Steele SJ, Bahadur G, Shenfield MB, Steele JW. Sperm banking. Assist Reprod Rev 1995;5:115-9.
17. Braken BR, Smith KD. Is semen cryopreservation helpful in testicular cancer? Urology 1980;60:581-3.
18. Aboulgher MA, Mansour RT, Serour GI, Amin YM. The role of intracytoplasmic sperm injection in the treatment of borderline semen. Hum Reprod 1995;10:2829-30.
19. Telfer P. Sexual transmission of hepatitis C virus. Haemophilia 1995; 1(4):15-18.
20. Fried MW, Shindo M, Fong TL, Fox PC, Hoofnagle JH, Di Bisceglie AM. Absence of hepatitis C viral RNA from saliva and semen of patients with chronic hepatitis C. Gastroenterology 1992;102(4 Pt 1):1306-08.
21. Hsu HH, Wright TL, Luba D, Martin M, Feinstone SM, Garcia G, et al. Failure to detect hepatitis C virus genome in human secretions with the polymerase chain reaction. Hepatology 1991;14(5):763-67.
22. Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AK, Fielding A, Briggs EM, et al. Hepatitis B transmission from contaminated cryopreservation tank. Lancet 1995;346:137-40.
23. Ballou JD. Potential contributation of cryopreserved germplasm to the preservation of genetic diversity and conservation of endangered species in captivity. Cryobiology 1992;29:19-25.
24. Watson PF. An objective method for measuring fluorescence of individual sperm cells labelled with 1-anilinonaphthalene-8-sulphonate (ANS) by means of photomicrography and densitometry. J Microsc 1979;117(3):425-29.
25. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 1949;164(4172):666.
26. Smith AU, Polge C. Survival of spermatozoa at low temperatures. Nature. 1950 ;166 (4225):668-69.

27. Stewart DL, Melrose DR, Wilson WR. The effect of reducing the quantity of egg yolk in bovine semen diluents. Vet Rec 1950; 62(44):617-18.
28. Arakawa T. Advanced drug delivery reviews 2001;46(1-3):307-26.
29. Ivanov IT. Rapid method for comparing the cytotoxicity of organic solvents and their ability to destabilize proteins of the erythrocyte membrane. Pharmazie 2001;56(10):808-09.
30. Kelley FD, Phelan RM, Levin RL. Controlled-rate liquid N2-microwave biological freeze—thaw device. Cryobiology 1982; 19(4):372-91.
31. Yamashiro H, Narita K, Sugimura S, Han YJ, Sugawara A, Morohaku K, et al. Trehalose enhanced the freezability of Poodle dog sperm collected by an artificial vagina (AV). Anim Reprod Sci 2007;102(1-2):165-71.
32. Hammitte DG, Walker DL, Williamson RA. Concentration of glycerol required for optimal survival and in vitro fertilizing capacity of frozen sperm is dependent on cryopreservation medium. Fertil Steril 1988;4:680-87.
33. Cohen J, Garrisi GJ, Congeda-Ferrara TA, Kieck KA, Schimmel TW, Scott RT. Cryopreservation of single human spermatozoa. Hum Reprod 1997;12(5):994-1001.
34. Critser J, Huse-Benda AR, Aaker DV, Ameson BWA, Ball GD. Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil Steril 1988;50(2):314-20.
35. Jeyendran RS, Van der Ven MM, Kennedy W, Perez-Pelaez M, Zaneveld LJD. Comparison of glycerol and a zwitterion buffer system as cryoprotective media for human spermatozoa. J Androl 1984a;5:1-6.
36. Jeyendran RS, Van der Ven MM, Kennedy W, Perez-Pelaez M, Zaneveld LJD. Effect of glycerol and cryopreservation on oocyte penetration by human spermatozoa. Andrologia 1985;17:241-48.
37. Puris GS, Weidel L. A comparative study of buffer systems as cryoprotectants for human spermatozoa. Fertil Steril 1986;46(1):147-49.
38. Graham EF. Fundamentals of the preservation of spermatozoa. In integrity of frozen spermatozoa: Proceedings of a round-table conference, Washington DC, 1976;Washington DC, National Academy of Sciences 1978;4-44.
39. Silva AL, Yamasaki R, de Sala MM, Cabera M, Da G, de Sa MF. The addition of fructose or sodium citrate does not improve recovery rates of cryopreserved human spermatozoa. Int J Fertil Menopausal Stud 1996;41(3):504-09.
40. Mahadevan M, Trouson AO. Effect of cryoprotective media and dilution methods on the preservation of human spermatozoa. Andrologia 1983;15(4):355-66.
41. Vincent JS, Viudes-de-Castro MP. A sucrose-DMSO extender for freezing rabbit semen. Reprod Nutr Dev 1996;36(5):485-92.
42. Zavos PM, Correa JR, Zar makoupis-Zavos PN. Evaluation of techniques for the cryopreservation of washed spermatozoa: Comparison between Ham's F-10 and Test-yolk media. Tohoku J Rxp Med 1998;184(4):277-84.
43. Hammadeh ME, Greiner S, Rosenbaum P, Schmidt W. Comparison between human sperm preservation medium and TEST-yolk buffer on protecting chromatin and morphology integrity of human spermatozoa in fertile and subfertile men after freeze-thawing procedure. J Androl 2001;22(6):1012-18.
44. Aitken RJ, Buckingham D, Brindle J, Gomez E, Baker G, Irvine DS. Analysis of sperm movement in relation to the oxidative stress created by leucocytes in washed sperm preparations and seminal plasma. Hum Reprod. 1995;10:2061-71.
45. Barratt CL, Naeeni M, Clements S, Cooke ID. Clinical value of sperm morphology for in-vivo fertility: comparison between World Health Organization criteria of 1987 and 1992. Hum Reprod. 1995;10(3):587-93.
46. Oehninger S, Kruger T. The diagnosis of male infertility by semen quality. Clinical significance of sperm morphology assessment. Hum Reprod. 1995; 10(5):1037-38.
47. Rogers BJ, Bentwood BJ, Van Campden H, Helmbrecht G, Soderdahl D, Hale RW. Sperm morphology assessment as an indicator of human fertilizing capacity. Androl 1983;4:119-25.
48. Yang YS, Chen SU, Ho HN, Chen HF, Chao KH, Lin HR, et al. Correlation between sperm morphology using strict criteria in original semen and swim-up inseminate and human in vitro fertilization. Arch Androl 1995;34:105-13.
49. Ombelet W, Pollet H, Bosmans E, Vereecken A. Results of a questionnaire on sperm morphology assessment. Hum Reprod. 1997;12:1015-20.
50. Francavilla F, Fromano R, Santucci R, Poccia G. Effect of sperm morphology and motile sperm count on outcome of intrauterine insemination in oligospermia and/or asthenospermia. Fertil Steril. 1990;53: 892-97.
51. Lindheim SR, Barad DH, Zinger M, Witt B, Amin H, Cohen B, et al. Abnormal sperm morphology is highly predictive of pregnancy outcome during controlled ovarian hyperstimulation and intrauterine insemination. J Assist Reprod Genetics. 1996;13:569-72.
52. Kobayshi T, Jinno M, Sugimura K, Notsawa S, Sugiyama T, Iida E. Sperm morphology assessment based on strict criteria and in vitro fertilization outcome. Hum Reprod. 1991;6:983-86.
53. Grow D, Oehninger S. Strict criteria for the evaluation of human sperm morphology and its impact on assisted reproduction. Andrologia 1994;27:325-33.
54. Lim CC, Lewis SE, Kennedy M, Donnelly ET, Thompson W. Human sperm morphology and in vitro fertilization: sperm tail defects are prognostic for fertilization failure. Andrologia 1998;30:43-47.
55. Haidl G. Andrologische Diagnostik Heute. Fortschr Med 1996;114:465-69.

56. Ibrahim A, Mousa MAA, Dedersen H. Sperm chromatin heterogeneity as an infertility factor. Arch Androl. 1988;21:129-33.
57. Jeyendran RS, Acosta VC, Land S, Coulam CB. Cryopreservation of human sperm in a lecithin-supplemented freezing medium. Fertil Steril 2008 Feb 2 [Epub]
58. Ollero M, Blanco TM, López-Pérez MJ, Cebrian Pérez JA. Surface changes associated with ram sperm cryopreservation revealed by counter-current distribution in an aqueous two-phase system effect of different cryoprotectants, J Chromatogr B Biomed Appl 1996;680(1-2):157-64.
59. Ollero M, Perez-Pe R, Muiño-Blanco T, Cebrian-Perez JA. Improvement of Ram Sperm Cryopreservation Protocols Assessed by Sperm Quality Parameters and Heterogeneity Analysis. Cryobiology 1998;37(1):1-12.
60. Aisen E, Quintana M, Medina V, Morello H, Venturino A. Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. Cryobiology 2005;50(3):239-49.
61. Yamashiro H, Han YJ, Sugawara A, Tomioka I, Hoshino Y, Sato E. Freezability of rat epididymal sperm induced by raffinose in modified Krebs–Ringer bicarbonate (mKRB) based extender solution", Cryobiology 2007;55(3):285-94.
62. Tournaye H, Camus M, Goossens A, Liu J, Nagy P, Silber S, et al. Recent concepts in the management of infertility because of non-obstructive azoospermia. Hum Reprod 1995;10 (1):115-19.
63. Lahav-Baratz S, Rothschild E, Grach B, Koifman M, Shiloh H, Ishai D, Dirnfeld M. The value of sperm pooling and cryopreservation in patients with transient azoospermia or severe oligoasthenoterato-zoospermia. Hum Reprod. 2002;17(1):157-60.
64. Geens M, Goossens E, De Block G, Ning L, Van Saen D, Tournaye H. Autologous spermatogonial stem cell transplantation in man: current obstacles for a future clinical application. Hum Reprod Update. 2008;14(2):121-30. Epub 2008 Jan 10.
65. Brougham MF, Wallace WH. Subfertility in children and young people treated for solid and haematological malignancies. Br J Haematol 2005;131(2):143-55.
66. Counsel M, Bellinge R, Burton P. Vitality of oligozoospermic semen samples is improved by both swim-up and density gradient centrifugation before cryopreservation. J Assist Reprod Genet 2004; 21(5):137-42.
67. Crabbé E, Verheyen G, Tournaye H, Van Steirteghem A. Freezing of testicular tissue as a minced suspension preserves sperm quality better than whole-biopsy freezing when glycerol is used as cryoprotectant. Int J Androl 1999;22(1):43-48.
68. Ohta H, Sakaide Y, Wakayama T. Long-term preservation of mouse spermatozoa as frozen testicular sections. J Reprod Dev 2008;54(4):295-8. Epub 2008 May 13.
69. Sahu GK, McNearney T, Evans A, Turner A, Weaver S, Huang JC, Baron S, Paar D, Cloyd MW. Transient or occult HIV infections may occur more frequently than progressive infections: changing the paradigm about HIV persistence. Arch Virol Suppl 2005;(19):131-45.
70. Crha I, Ventruba P, Zakova J, Huser M, Kubesova B, Hudecek R, Jarkovsky J. Survival and infertility treatment in male cancer patients after sperm banking. Fertil Steril 2008 Jun 11. [Epub ahead of print]
71. Williams RS, Alderman J. Predictors of success with the use of donor sperm.Am J Obstet Gynecol 2001 Aug;185(2):332-37.
72. Bleich JD. Sperm banking in anticipation of infertility. Tradition 1995;29(4):47-60.
73. Whitney S, Mian P. Life after death? Ethical questions raised after a request for postmortem sperm retrieval in the emergency department. J Emerg Nurs 1998;24(6):492-94.
74. Shefi S, Raviv G, Eisenberg ML, Weissenberg R, Jalalian L, Levron J, Band G, Turek PJ, Madgar I. Posthumous sperm retrieval: analysis of time interval to harvest sperm. Hum Reprod 2006;21(11):2890-93. Epub 2006 Sep 7.
75. Bruce SD. Sperm and egg banking give patients hope for parenthood after cancer treatment. ONS Connect 2007;22(6):17.
76. Møller H, Skakkebæk NE. Risk of testicular cancer in subfertile men: case-control study. BMJ 1999;318(7197):1556.
77. Lass A, Akagbosu F, Abusheikha N, Hassouneh M, Blayney M,Avery S, et al.A program of semen cryopreservation for patients with malignant disease: lessons from eight years' experience. Hum Reprod 1998;11:3256-61.
78. Meirow D, Schenker JG. Cancer and male infertility. Hum Reprod 1995;10:2017-22.
79. Agarwal A. Semen banking in patients with cancer: 20-year experience. Int J Androl 2000;23(2):16-9.
80. Aareleid T, Sant M, Hédelin G. Improved survival for patients with testicular cancer in Europe since 1978. Eur J Cancer 1998;34(14):2236-40.
81. Saito K, Suzuki K, Iwasaki A, Yumura Y, Kubota Y. Sperm Cryopreservation before cancer chemotherapy helps in the emotional battle against cancer. Cancer 2005;104(3):521-24.
82. Meguro A, Muroi K, Miyoshi T, Matsuyama T, Mori M, Nagai T, Ozawa K. Sperm cryopreservation in patients with hematologic malignancies. Int J Hematol. 2008 Aug 12. [Epub ahead of print]
83. Kopeika J, Kopeika E, Zhang T, Rawson DM, Holt WV. Effect of DNA repair inhibitor (3-aminobenzamide) on genetic stability of loach (Misgurnus fossilis) embryos derived from cryopreserved sperm. Theriogenology 2004;61(9):1661-73.
84. Practice Committee of the Society for Assisted Reproductive Technology; Practice Committee of the American Society for Reproductive Medicine. Guidelines for development of an emergency plan for in vitro fertilization programs. Fertil Steril. 2008 Apr;89(4):793-5. No abstract available.

APPENDIX: CRYOPRESERVATION PROTOCOL

Equipment

Counting chamber
5 µl micropipette
Aliquot mixer
Vortex
– 20 °C Freezer
LN2 container with racks
Sterile specimen container
Sterile 15 mL Centrifuge tubes with caps
Sterile Serological Pipettes (1 mL, 2 mL and 5 mL capacity)
Sterile Cryovials (1 mL and 2 mL capacity)
Colored Cryomarkers
Test tube racks (for 15 mL test tubes)
Cryovial racks
Stainless steel canes for cryovials
Plastic Cryosleeves
Cryogloves
Latex gloves
37 °C Incubator
Ln2 from supplier
Eosin-nigrosin stain
Microslide
Cover slips
Sperm washing media
Makler chamber

Reagents

Freezing medium
Sperm washing media

Procedure

- All procedures are done using sterile techniques.
- All client depositors are given information on the future usefulness of his frozen semen specimen.
- During the collection of the specimen, important information (e.g. abstinence time, method of collection, time of collection) is noted down and labeled.
- Collected semen specimen is then incubated at 37°C for a minimum of 20 minutes to allow liquefaction. If liquefaction does not occur after incubation, it can be manipulated by pipetting it up and down.
- At the same time, a bottle of the freezing medium is taken from the – 20 °C is allowed to thaw at room temperature.
- After the semen has liquefied, draw the semen specimen into a 5 mL pipette.
- Volume is measured and any unusual consistency before delivering it into a labeled sterile 15 mL conical tube.
- Automated semen analysis is then used to determine the concentration, motility, curvilinear velocity, linearity and amplitude of lateral head movement. Manual assessment is followed for concentration, motility and presence of round cells.
- Presence of round cell is quantified and Endtz test is conducted of >5 round cells/hpf are seen under wet preparation. Any bacteria or foreign cells should be noted.
- Within an hour of specimen collection, add an aliquot of freezing medium equaling 4 times the original specimen volume to the centrifuge tube with a sterile pipette and gently mix it on an aliquot mixer. Volume should not exceed 1 mL.
- This should be repeated 3 times, until the volume of freezing media added is equal to original specimen volume.
- Cryovials are labeled accordingly and specimen + freezing media is distributed into it using a 1 or 2 mL sterile serological pipette.
- Left over aliquot of the cryodiluted specimen should be inspected for motility by using a counting chamber and a microscope. Percentage of motility should be documented.
- The labeled cryovials are placed into a plastic freezing rack along with canes and cryosleeves. A maximum of 2 cryovials are placed into the bottom slots of the canes upside down. The cryosleeves together with the canes and vials are placed bottom side up into a – 20°C freezer for 8 minutes.
- After 8 minutes, remove the rack and canes from the freezer and placed them, bottom side up, into the liquid nitrogen vapor (14-17 cm away from the liquid nitrogen) for a minimum of 2 hours.
- After a minimum of 2 hours in the liquid nitrogen vapors, canes are turned upside down and immersed into the liquid nitrogen.
- After a minimum of 24 hours in liquid nitrogen, thaw a test aliquot (usually 20 to 50 µl left in an extra cryovial) for estimation of survival rates.
- For thaw, using cryogloves and protective goggles, the cane containing the vial is removed and the vial is placed in the 37°C incubator for 20 minutes.

- Mix the vial well and analyze the cryopreserved specimen for count, motility, curvilinear velocity, linearity, and amplitude of lateral head movement using the automated analyzer followed by the manual assessment.
- Cryosurvival results are recorded.

Procedure (Surgically Retrieved Spermatozoa)

1. Cryopreservation worksheet labeled with the patient name, diagnosis and LN2 container and rack. If there is more than one specimen, then have a worksheet for each specimen.
2. Every time a laboratory receives the notice that an aspirate is ready, take one bottle of freezing media and put into the 37 °C incubator to thaw.
3. A technologist will go to the surgery room to retrieve the specimen(s). Keep the vials warm in your hands.
4. Normally, two to three specimens will arrive from surgery. Make sure patient name and specimen number are written.
5. Using sterile technique measure the volume of each specimen and record on the appropriate cryopreservation worksheet.
6. Centrifuge the specimen in the original container for 5 minutes at 1600 rpm.
7. Label 3 sterile 15 mL conical centrifuge tubes with the patient's name, specimen number.
8. Transfer the supernatant of specimen one and specimen 2 and if there is specimen 3 also in the "supernatant" tube. Both supernatants are now combined into one tube.
9. Add 0.5 mL HTF (sperm washing media) to each pellet to resuspend. Mix gently.
10. Transfer a drop of each specimen into a pre-labeled conical cup for semen analysis.
11. Perform the following on each specimen aspirate: Regular manual semen analysis, Endtz test (Leukocytospermia test), Eosin-nigrosin stain procedure, Tygerberg's strict criteria form morphology evaluation..
12. Always notify if the surgeon found no motile sperm on the wet prep.
13. Within one hour of specimen collection, add an aliquot of freezing medium equal to 25% of the resuspended aspirate volume to the centrifuge tube with a sterile pipette.

    Note: Since the resuspended volume for each aspirate is 0.5 mL then divide 0.5 by 4 to obtain 0.13. Therefore, add 0.13 mL of freeze media 4 times to the specimen.
14. Gently rock the specimen(s) with the freezing media for 5 minutes on an aliquot mixer.
15. Repeat steps 13 and 14 three times or until the volume of freezing media added is equal to the specimen volume in step 9.
16. Centrifuge the tube labeled "supernatant" for 5 minutes at 1600 rpm.
17. Remove the supernatant from the "supernatant" tube. Resuspend the pellet with 0.5 mL of HTF (sperm washing media). Mix gently. Remove one drop for a semen analysis.

    Note: If no motile sperm is found in the "supernatant" tube, then do not start freezing. If motile sperm is found, then freeze as in steps 13 to 15.

    During the mixing steps above, use appropriately colored cryomarkers to label 2 mL cryovial and canes. The volume added to the vial should not exceed 1.8 mL per vial.
18. Label an additional 1.0 mL cryovial as in for each specimen. This will contain a leftover aliquot of the cryodiluted specimen to be assessed of cryosurvival 24 hours after freezing in LN2.
19. A visual inspection should therefore be made of the cryodiluted specimen for motility. A manual motility can be done using a counting chamber or a Makler chamber and a 2-phase microscope. The percent motility should be documented on the cryopreservation worksheet under cryodilution motility.
20. Distribute the well-mixed, cryodiluted semen into pre labeled vials using a 1 or 2 mL sterile serological pipette. Add at least 0.2 mL to the smaller 1.0 mL cryovial.
21. Place labeled vials into a plastic freezing rack along with canes and cryosleeves put into a – 20° C freezer for 8 minutes. Do not open the freezer in any circumstance during this incubation.

    Note: Exposure to freezing conditions should occur within 1.5 hours of specimen collection.
22. After the 8 minute incubation, remove the rack and canes from the 20° C freezer. Place a maximum of 2 cryovials into bottom slots of canes up side down. Put into cryosleeves.

23. After minimum 2 hours incubation in liquid nitrogen vapors, turn cases up side down, immerging them into liquid nitrogen.
24. After a minimum of 24 hours in liquid nitrogen, thaw the aliquot in the 1.0 mL cryovial.
25. Using cryogloves, remove cane containing the vial and snap it out. Loosen the cap and place in the 37° C incubator for 20 minutes.
26. Mix the vial well and analyze manually.
27. Record the cryosurvival area of the cryopreservation worksheet.
28. Assess cryo-survival in the formal:

$$\frac{\text{Post - thaw specimen motility \%}}{\text{Pre - freeze specimen motility \%}} \times 100$$

Precautions: Sterile techniques should be used throughout specimen processing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, may be toxic to sperm. Therefore, care should be taken to prevent contamination of the specimen with latex or talc. Vinyl gloves are an available alternative.

SEMEN CRYOPRESERVATION REPORT

Name: _____ Clinic: _____ Bank ID: _____

	Specimen A	*Specimen B*	*Specimen C*
Date			
Abstinence time			
Volume (ml)			
Pre cryopreservation			
Motility (%)			
Percent motile			
Velocity(μ/sec)			
Linearity (%)			
ALH (μ)			
Count (10^6/ml)			
Motile sperm (106/ml)			
Cryosurvival (%)			
Motility (%)			
Percent motile			
Velocity(u/sec)			
Linearity (%)			
ALH (μ)			
Motile sperm (10^6/ml)			
Volume/vial(ml)			
Motile sperm/vial(10^6)			
#vials			
Total motile sperm (10^6)			
#inseminations			
IVE-IVF attempts			

Comments

SEMEN CRYOPRESERVATION REPORT

Name: Clinic: Bank ID:

Specimen
Date
Cont. Time
Volume

Precryopreservation

Motility
- Percent motile
- Velocity(u/sec)
- Linearity
- ALH(u)
- Count (m/ml)
- Total motile sperm (m/ml)

Post-thaw cryosurvival

Motility
- Percent motile
- Velocity(u/sec)
- Linearity
- ALH(u)
- Count(m/ml)
- Total motile sperm (m/ml)

#vials
Total motile sperm (m)
#inseminations
IVF attempts

Comments

CHAPTER 22

Donor Insemination

Natachandra Chimote, Meena Chimote

Infertility, though not life-threatening, causes intense mental agony and trauma that can only be best described by infertile couples themselves. There are no detailed figures of the extent of infertility prevalent in India but a multinational study carried out by WHO (Diagnosis and treatment of infertility, editor P Rowe and EM Vikhlyaeva, 1988) that included India, places the incidence of infertility between 10 and 15%. Out of a population of one billion Indians, an estimated 25% (250 million individuals) may be conservatively estimated to be attempting parenthood at any given time; by extrapolating the WHO estimate, approximately 13 to 19 million couples are likely to be infertile in the country at any given time.

About 10% of infertile couples, however, need medical intervention involving the use of ART (Assisted Reproductive Technologies) procedures such as IVF (*In virto* Fertilization) or ICSI (Intracytoplasmic Sperm Injection).[1] Such advanced treatment is expensive and not easily affordable to the majority of the Indians. Further, the successful practice of ART requires considerable technical expertise and expensive infrastructure. Moreover, the success rate of any ART procedure is below 30% under the best of circumstances. Infertility, especially in our country, also has far-reaching societal implications. Therefore, with the rapidly increasing use of ART in our country, it has become imperative to ensure safety and have safeguards against their possible misuse.

Male infertility may be due to diminished spermatogenesis or defects in the fertilizing potential of sperm. In either case, the severity of the diminished fertility and the available treatment options are variable. Although the advent of intracytoplasmic sperm injection (ICSI) has allowed homologous conception in many couples with severe male-factor infertility, the increased cost, possible risks, and the invasiveness of IVF and ICSI have precluded ICSI therapy in most of the couples in India. Additionally, most cases of azoospermia are still untreatable with ICSI.[2] Therefore, therapeutic insemination of donor sperm remains the chosen therapy of many couples with male-factor infertility.

INTRACYTOPLASMIC SPERM INJECTION (ICSI) VS DONOR INSEMINATION (DI)

Since the first pregnancy from *in vitro* fertilization (IVF) in 1978 and with even advanced technique like intracytoplasmic sperm injection (ICSI)[3] reported in 1992, has revolutionized the treatment of severe male factor infertility.[4-7] Fertilization rates with ICSI are so high that female age, with its attendant impact on the quality and number of eggs retrieved, has become the limiting factor in pregnancy rates for couples. Parameters of semen quality, in contrast, appear irrelevant as long as viable spermatozoa are available.[4,6] IVF-ICSI therefore provides a new option to couples who previously were restricted to donor insemination (DI) or adoption to obtain a child DI is often regarded unfavorably in Western society.

An American survey of men in infertile couples found that only 43% would consider using DI and, in a matched sample without infertility, only 15% of men would contemplate it.[8] In a Spanish cohort of couples choosing DI, were more passive and more apprehensive about

pregnancy than their wives.[9] Such studies have not been carried out so far in India, but the overall feeling about DI is not so unfavorable. Couples who use DI also have to confront difficult decisions about whether to tell their child about his or her genetic origins.[10,11] For the minority who choose to be open,[11,12] information about the donor remains quite limited in most countries. However, the impact of the availability of ICSI on infertile men over acceptance of DI has not been studied so far.

Unfortunately, infertility treatment in India is one of the most neglected and looked down upon medical fields for wrong reasons wherein no insurance coverage has been given to the infertile couples. The preliminary findings suggest we may be creating a three-tiered system of care for men with severely limited fertility:

- A genetic child if they can afford to pay for one, and otherwise the options of a child who is not biologically related, or living without children.

Economic forces already limit the availability of assisted reproductive technology, and the success of IVF-ICSI merely heightens the contrasts between rich and poor couples. DI is obviously the most inexpensive way for these couples to conceive in the western world. But in India, DI is less costly and not much time-consuming than a typical public agency adoption. Obviously, the cost differential between IVF-ICSI and DI is less in some nations that include assisted reproduction under a national health care system and in such circumstances, the couples' preferences for types of treatment should be studied. A strikingly higher percentage of men in India who choose DI, do not give importance of biological parenthood. And they volunteer donor sperm as a rationale for adjusting to the economic necessity and not as the primary reason for choosing the donor program.

In case of women, both IVF-ICSI and DI offer the chance to experience pregnancy and to bear a genetically related child. In fact, it is a paradox that the wife, who typically has normal fertility, undergoes most of the medical risk and discomfort of an IVF-ICSI cycle. A greater percentage of women than men cited the increased health risks of IVF to the female partner or the fear of having a genetically abnormal child as reasons for choosing DI. Therefore, a few women prefer IVF-ICSI before considering DI. If their first choice of infertility treatment fails, women also are more open than men to adoption.

The main advantage of DI is that it enables the couple to achieve pregnancy even though the husband is not the biological father. However, the possible transmission of diseases from the donor to the future child and the risk of consanguinity, constitute some drawbacks that must be brought to the notice of the patients. It is necessary to get the informed consent of both the partners after they are counseled about the possible psychological conflict they may face later in their life with the knowledge that one of them is not the biological parent of their child. DI is an ethically acceptable procedure provided there is a medical indication and psychological confirmation for its use.

Also, the normal conditions of anonymity and screening of the donor must be met and only frozen sperm samples that have passed appropriate quarantining for infectious diseases such as HIV, hepatitis B and C, and syphilis should be used.

Therapeutic donor insemination (TDI) may be employed to achieve conception where appropriate indications exist. The clinical procedures should take into account the age and health status of the recipient. The ICMR has published requirements for the screening and testing of donors of human cells, tissues, and cellular and tissue-based products (HCT/Ps), which are included here. It is the responsibility of all clinics to know the regulations of their individual states and local municipal corporations and to comply with those standards.

Indications for Considering Donor Insemination

- The male partner has azoospermia, severe oligozoospermia, or other significant sperm or seminal fluid abnormalities.
- The male partner has ejaculatory dysfunction.
- The male partner demonstrates significant male factor infertility (i.e. significant oligoasthenozoospermia or prior failure to fertilize after insemination *in vitro* and intracytoplasmic sperm injection (ICSI) is not elected or feasible).
- The male partner has a significant genetic defect or the couple previously has produced an offspring affected by a condition for which carrier status cannot be determined.
- The male partner has a sexually transmissible infection that cannot be eradicated.
- The female partner is Rh-negative and severely Rhisoimmunized, and the male partner is Rh-positive.
- Females without male partners.

Evaluation of the Male Partner

A. The male partner in any couple that requests TDI should have completed an appropriate clinical evaluation. Medical records should be reviewed before performing the insemination procedure. If appropriate, alternative treatments should be discussed with the couple.
B. Human immunodeficiency virus (HIV) testing of the male partner is recommended strongly to address potential medical/legal issues that could arise if his partner seroconverts during or after TDI. In addition, if the male partner is HIV infected, he should be referred to an appropriate infectious disease unit for counseling on safe sex practices for preventing HIV transmission, on treatment options, and on other issues concerning HIV disease. A positive HIV test result for the male partner should not be used as an exclusionary criterion for treatment of a couple with TDI, provided that the semen to be used derives from an HIV-negative donor. Current ICMR guidelines do not entirely preclude the use of an HIV positive directed donor. However, in the opinion of ICMR, HIV-positive directed donors should not be used, because the risk of viral transmission cannot be eliminated completely.
C. Testing for other STIs similar to that recommended for the female partner is encouraged.

Evaluation of the Female Recipient

A. Routine medical and reproductive history should be obtained according to the standards that are applied to women anticipating pregnancy. Abnormalities detected from history or physical examination may require more detailed evaluation and treatment before proceeding with insemination.
B. A complete general physical examination should be performed, including a pelvic examination.
C. Standard preconception screening, testing and counseling:
   Recommended tests include:
   a. Blood type, Rh factor, and antibody screen.
   b. Rubella and varicella titers. Vaccination should be offered if the individual is not immune to either virus.
   c. *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.
   d. HIV-1 and -2 testing should be performed to address potential medical/legal complications that could arise if the recipient seroconverts during or after treatment. In addition, if the female recipient is found to be HIV infected before treatment, she should be referred to an appropriate infectious disease unit for counseling on issues concerning HIV disease, including reproductive issues such as safe sex practices for preventing HIV transmission to uninfected partners and treatment options to reduce the probability of transmission to her child.
      A positive HIV-1 or -2 test of the female recipient should not be used as an exclusionary criterion for treatment with TDI as long as the couple makes an informed decision following counseling and agrees to comply with recommended clinical management for the positive HIV status during pregnancy.
   e. Serologic test for syphilis.
   f. Hepatitis B surface antigen.
   g. Hepatitis B core antibody (IgG and IgM).
   h. Hepatitis C antibody.
   i. Cytomegalovirus (CMV) antibody (IgG and IgM). For women who test positive for active infection (positive urine or throat culture or paired serum samples demonstrating a fourfold rise in IgG antibody and IgM antibody at least 30% of the IgG level), attempts to conceive should be postponed until they no longer exhibit active infection, owing to the risk of transmitting the infection to their fetus and the serious potential consequences of fetal CMV infection.
   j. Human T-cell lymphotropic virus (HTLV) Type I and II may also be obtained at the discretion of the clinician in the appropriate clinical setting.
D. *Documentation and timing of ovulation:* Women with regular cyclic menses are assumed to be ovulating. When doubt exists, an index of ovulation, such as serum progesterone level, basal body temperature, luteinizing hormone (LH) surge detection and ultrasound monitoring of follicular maturation, may be used to document ovulation. Appropriate timing of the insemination procedure optimizes chances for success.
E. *Evaluation for possible tubal or peritoneal abnormalities:* Patients who fail to conceive after three to four well timed inseminations may be candidates for hysterosalpingography (HSG), laparoscopy, or other appropriate tests to detect possible causes for their failure to conceive. Pretreatment HSG or laparoscopy may be indicated by the history and/or physical findings.

F. Informed consent should be obtained from the patient (and her partner, if applicable).

Selection of Donors: ICMR Guidelines for Donor Sperm

At present we in India do not have any guidelines or legislation for the ethical practice of ART. Besides, India has been known to play host to visiting foreign *in vitro* fertilization (IVF) experts to carry out such procedures that have been banned in their home country. These clinics are capitalizing on the intense desire of infertile couples to become parents. Therefore, ICMR has set guidelines for accreditation, supervision and regulations of ART clinics and clinics practicing first line treatment of infertility, i.e. only IUI. Even as the world debates the authenticity of the first cloned baby, the Indian Council for Medical Research (ICMR) has drafted guidelines for regulation of Assisted Reproductive Technology (ART). The draft guidelines have been submitted to the Union Health Ministry.

As per the guideline of ICMR given chapter three in their guidelines, following are the selection criteria for a semen donor:

- The main qualities to seek in selecting a donor for TDI are an assurance of good health status and the absence of genetic abnormalities. The individual must be free of HIV and hepatitis B and C infections, hypertension, diabetes, sexually transmitted diseases, and identifiable and common genetic disorders such as thalassemia and sickling (ICMR: 3.6.1).
- The age of the donor must not be below 21 or above 45 years (ICMR: 3.6.2) because increased male age is associated with a progressive increase in the prevalence of aneuploid sperm.
- An analysis must be carried out on the semen of the donor, preferably using a semen analyzer, and the semen must be found to be normal according to WHO method manual for semen analysis, if intended to be used for ART (ICMR: 3.6.3) Selection of donors with established fertility is desirable but not required.

Semen Testing

- The sample should be examined within 1 to 2 hours after ejaculation into a sterile container. The criteria used to judge the normality of the sample can vary among laboratories. There are no uniformly accepted standards, but, in general, the minimum criteria for normal semen quality can be applied.
- The blood group and the Rh status of the individual must be determined and placed on record.[3,4,6]
- Other relevant information in respect of the donor, such as height, weight, age, educational qualifications, profession, color of the skin and the eyes, record of major diseases including any psychiatric disorder and the family background in respect of history of any familial disorder, must be recorded in an appropriate proforma.[3,5,6]

Genetic Evaluation

Genetic screening for heritable diseases should be performed in potential sperm donors. Testing for cystic fibrosis carrier status should be performed on all donors. Other genetic testing should be performed, as indicated by the donor's ethnic background in accordance with current recommendations, after obtaining a proper family history.

There is no method to completely ensure that infectious agents will not be transmitted by TDI, but the following guidelines, in addition to adequate history taking and exclusion of individuals at high risk for HIV and other STIs, should dramatically reduce these risks prior to use of the sperm.

The List of Tests

a. HIV-1 and -2.
b. Hepatitis C antibody.
c. Hepatitis B surface antigen.
d. Hepatitis B core antibody (IgG and IgM).
e. Serologic test for syphilis.
f. CMV (IgG and IgM). Men who test positive for active infection (positive urine or throat culture or paired serum samples demonstrating a fourfold rise in IgG antibody and IgM antibody at least 30% of the IgG level) should be excluded. Because CMV is so common, insemination with semen from a CMV-seropositive man (without active infection) is permissible when the female partner is also CMV seropositive. Although the practice is not entirely without risk, because there are many strains of CMV and superinfection is possible, the associated risk of newborn CMV infection is approximately 1%, and such infants appear to have no significant illness or other abnormality.

Managing Laboratory Results

a. A positive test should be verified before notifying the potential donor. If a test is confirmed positive, the

individual should be referred for appropriate counseling and management.

b. If testing is negative, semen samples may be collected and prepared for cryopreservation.
c. After donation, anonymous donor specimens must be quarantined for a minimum of 180 days. The donor must be retested after the required quarantine interval, and specimens may be released only if the results of repeat testing are negative.
d. Screening and testing of donors for STDs and genetic risk factors may change over time as tests improve and new tests become available. Therefore, samples of sperm that are cryopreserved and stored for periods of time may not meet existing testing standards at the time they are released for use. In such instances, every effort should be made to have the donor tested in accordance with current standards. In situations where the donor is not available or refuses such additional testing, the sample(s) may be released provided that the recipient is informed that the specimen does not meet current screening and testing guidelines, and is informed, what tests have not been performed, and is counseled regarding the clinical implications of the missing information.

Responsibility of ART Center

Moreover, ICMR also has laid down certain guidelines pertaining to selection of semen donors and responsibilities of ART clinics.

- Use of sperm donated by a relative or a known friend of either the wife or the husband shall not be permitted.
- It will be the responsibility of the ART clinic to obtain sperm from ***appropriate banks***; neither the clinic nor the couple shall have the right to know the donor identity and address, but both the clinic and the couple, however, shall have the right to have the fullest possible information from the semen bank on the donor such as height, weight, skin color, educational qualification, profession, family background, freedom from any known diseases or carrier status (such as hepatitis B or AIDS), ethnic origin, and the DNA fingerprint (if possible), before accepting the donor semen.
- It will be the responsibility of the semen bank and the clinic to ensure that the couple does not come to know the identity of the donor.
- The ART clinic will be authorized to appropriately charge the couple for the semen provided and the tests done on the donor semen.
- Semen from two individuals must never be mixed before use, under any circumstance. (ICMR guidelines: 3,5,18)
- No owner, operator, laboratory director, or employee performing TDI can serve as a donor in that practice.
- Neither the patient's physician nor the individual performing the actual insemination can be the sperm donor.

COUNSELING

Psychological evaluation and counseling by a qualified mental health professional should be recommended strongly for all sperm donors. Psychological consultation should be required for individuals in whom there appear to be factors that warrant further evaluation. In cases of directed donation (known donors), psychological evaluation and counseling are recommended strongly for the donor and his partner, as well as for the recipient female and her partner, if applicable. The potential impact of the relationship between the donor and recipient should be explored. The psychological assessment should also address the potential psychological risks and evaluate for evidence of coercion (financial or emotional). It is important to ascertain whether the donor is well informed about the extent to which information about him might be disclosed and about any plans that may exist relating to future contact.

Regulations for Semen Banks (As per ICMR Guidelines)

- Either an ART clinic or a law firm or any other suitable independent organization may set-up a semen bank. If set-up by an ART clinic it must operate as a separate identity. (ICMR: 3.9.1.1)
- The bank will ensure that all criteria mentioned in Section 3.6 (Requirements for a sperm donor) are met and a suitable record of all donors is kept for 10 years after which, or if the bank is wound up during this period, the records shall be transferred to an ICMR repository (ICMR: 3.9.1.2)
- A bank may advertise suitably for semen donors who may be appropriately compensated financially (ICMR: 3.9.1.3)

- On request for semen by an ART clinic, the bank will provide the clinic with a list of donors (without the name or the address but with a code number) giving all relevant details such as those mentioned in Section 3.6. The semen bank shall not supply semen of one donor for more than ten successful pregnancies. It will be the responsibility of the ART clinic or the patient, as appropriate, to inform the bank about a successful pregnancy. The bank shall keep a record of all semen received, stored and supplied, and details of the use of the semen of each donor. This record will be liable to be reviewed by the accreditation authority (ICMR: 3.9.1.4)
- The bank must be run professionally and must have facilities for cryopreservation of semen, following internationally accepted protocols. Each bank will prepare its own SOP (Standard Operating Procedures) for cryopreservation (ICMR: 3.9.1.5)
- Semen samples must be cryopreserved for at least six months before first use, at which time the semen donor must be tested for HIV and hepatitis B and C (ICMR: 3.9.1.6)
- The bank must ensure confidentiality in regard to the identity of the semen donor (ICMR: 3.9.1.7)
- A semen bank may store a semen preparation for exclusive use on the donor's wife or on any other woman designated by the donor. An appropriate charge may be levied by the bank for the storage. In the case of non-payment of the charges when the donor is alive, the bank would have the right to destroy the semen sample or give it to a bonafide organization to be used only for research purposes. In the case of the death of the donor, the semen would become the property of the legal heir or the nominee of the donor at the time the donor gives the sample for storage to the bank. All other conditions that apply to the donor would now apply to the legal heir, excepting that he cannot use it for having a woman of his choice inseminated by it. If after the death of the donor, there are no claimants, the bank would have the right to destroy the semen or give it to a bonafide research organization to be used only for research purposes (ICMR: 3.9.1.8)
- All semen banks will require accreditation (ICMR:3.9.1.9)

As already mentioned, sperm banks where a complete assessment of the donor has been done, medical and other vital information stored, quality of preservation ensured, confidentiality assured, and strict control exercised by a regulatory body, must be set-up. ***Donor sperm would be made available only through such specialized banks/ centers*** (ICMR: 3.14.3)

Primary (Level 1A) infertility care units or primary (level 1 B) infertility care units engaged in ***IUI should obtain donor sperm sample only from accredited sperm bank*** (ICMR: 2.5.1).

Rights of an Unmarried Woman to DI

There is no legal bar on an unmarried woman going for DI. A child born to a single woman through DI would be deemed to be legitimate. However, DI should normally be performed only on a married woman and that, too, with the written consent of her husband, as a two-parent family would be always better for the child than a single parent one, and the child's interests must outweigh all other interests (ICMR: 3.16.4)

Where do We get Semen Donors?

Male infertility is an important issue, and donor insemination is an established and cost-effective form of treatment not only in India but also in the western world. The availability of donor spermatozoa is a limiting factor all over the world.[11] Though, there has been no study done in India till date, many clinics in the UK have reported a shortage of spermatozoa for donor insemination[13] and several groups have looked at the motivation of sperm donors.[11,13-15] For example, Liu et al (1995)[13] reported that 69% of potential sperm donors expect financial reward. Pedersen and co-workers [15] found that 8% of sperm donors in their cohort stated purely altruistic motives for donation, 32% purely financial, and 60% a combination of both. These studies and other studies[11] of UK sperm donors have shown clearly that the majority of men who donate spermatozoa in the UK are young single students who are motivated predominantly by payment. This is at odds with the HFEAs (Human Fertilization and Embryology Authority) stated recommendation that attempts should be made to recruit older donors from stable relationships who already have children.

Whether Semen Donor should be Paid?

We really do not know whether semen donors in India are paid but we know it for sure that the recipient has to pay for donor sperm sample which is obtained from sperm processing laboratories. Studies both in the UK and USA

have clearly indicated that payment for sperm donation is an important consideration, particularly among younger (student) donor groups,[11] although the co-existence of altruistic motives together with financial considerations is well documented. Moreover, a recent survey of UK centers licensed for infertility treatment involving donated spermatozoa showed that almost all centers (97%) paid donors, and that 88% of centers surveyed believed that they would lose at least 80% of donors if payment was to be withdrawn.[16]

It has been recognized that the social and ethical issues imposed by assisted reproduction techniques are both complex and controversial.[17] These issues have been shown to vary between countries, and also between religious groups among donors and recipients.[18] On this backdrop, the scenario in India is more complex as we have several religions and varied ethnicity. As many as 33 states and more than 1000 languages and dialects which make this issue of semen donor even more complicated. Surprisingly, although there are numerous studies of the attitudes of donors in the western world, there is little or no information in India regarding attitudes to payment among donors, recipients and the general public drawn from the same geographical area.

It has been found that financial incentives have a bearing on whether men will donate spermatozoa. If such payments are withdrawn, there is a possibility that the availability of spermatozoa for treatment may decline. It has been shown that students strongly favor the payment for donors (67% in favor, 29% against).[18] Since in the UK students represent the main source of donors, our data add weight to the concern already voiced by infertility centers that withdrawal of payment could have significant implications.[16] While the potential recipient group also favored the payment of donors (53 versus 39%), the general public were strongly against payment (39 versus 58%). This raises an interesting question not only from an ethical, but also from a social and religious perspective: if the majority of both donors and recipients is in favor of such payment, but the general public is not, whose view should prevail?

More than 50% decline has been observed in the number of semen donors in most of the countries when the legislation was passed by their respective governments to disclose the identity of the donors to the donor offsprings.[19] Hence, it is prudent approach of ICMR not to disclose identity of the donor in India, so that semen donors would be available easily.

Will Frozen Semen Sample for Donor Insemination Reduce the Pregnancy Rate?

The disadvantage of adhering to the guidelines is the reduced pregnancy rate associated with frozen semen. Cryopreservation is accompanied by damage to a variety of sperm cell organelles due to intracellular ice crystal formation, cellular dehydration and osmotic injury. The currently accepted cryobiological view is that there is no functional loss during proper storage at -196ºC in liquid nitrogen for indefinite periods.[20] The process of DNA fragmentation in spermatozoa progresses even after ejaculation.[21] Nevertheless, there is a paucity of controlled studies designed to detect reproductive performance of cryopreserved sperm as a consequence of storage length.[22] Although the quarantining of frozen semen likely has negligible impact on semen quality, the freezing and thawing process is usually associated with diminished viability, motility, and functional ability of the sperm.[23] Sperm susceptibility to cryodamage appears to vary between individuals and often between samples of a given donor.[24] Therefore, despite the preliminary screening of semen quality of potential semen donors, evaluation of samples before cryopreservation and quarantine, the quality of thawed donor semen can be variable. It also has been reported that significant differences occur between sperm banks in the quality of donor semen provided. Additionally, significant variations exist within a given sperm bank.

This prolongs the mean time to conception, thus increasing the number of exposures and the likelihood that a couple will drop out of treatment. In addition, lower pregnancy rates may mean lower quality of life for potential mothers. It is appropriate to reappraise the requirement for exclusive use of frozenthawed semen in light of new knowledge about risks and outcomes.

How Many Times Same Donor's Semen should be Used?

The decision to permit use of fresh semen for donor insemination should be made by policy makers based on a number of factors, one of which is cost-effectiveness. The FDA, CDC, and ASRM in USA have requested to reevaluate the guidelines with a view toward allowing the use of fresh semen by informed patients. This would improve the autonomy of women who would choose the use of fresh semen to improve their chances of pregnancy.

It is estimated that more than 3 million babies have been born using assisted reproductive technology (ART) during the past 30 years, with about 200,000 currently being born each year.[25] The total number of offspring from anonymous donor sperm, however, is not known, although there are reports of the multiple use of individual donor's sperm and, in particular, of one sperm donor being responsible for 100-200 pregnancies.[26] Ostensibly, ''safe'' or ''acceptable'' limits to the number offspring for each anonymous sperm donor have been introduced in many countries for the reason that the unrestricted use of anonymous donor sperm carries a greater risk of consanguinity.[27-30]

The National Library of Health defines consanguinity as, ''descent from a common ancestor; [where] a consanguineous couple is usually defined as being related as second cousins or closer''[31] and the Dictionary of Genetics defines it as ''Meaning between blood relatives; usually refers to inbreeding or incestuous matings.''[32] This is an important consideration as consanguineous mating introduces an increased risk of serious abnormality in offspring. For instance, it introduces an additional 2% risk of serious abnormality for offspring from first cousin marriages and an additional 10% risk with half-sibling pairings.[30] These safe limits to the number of offspring per donor, however, seem to have been arbitrarily set and vary considerably between countries.[29, 31] For example, despite a population difference of 233.6 million people, there is a limit of 10 offspring per donor in both the United Kingdom and the United States. Similarly, despite similar populations, France permits 5 offspring per donor, whereas the United Kingdom permits 10. Psychosocial and cultural factors, rather than medical–genetic calculations,[33,34] seem to have been the primary considerations in setting the limits for most countries.[29, 31] ICMR in India has proposed the number as 10 offspring per donor. With such a diverse ethnic, linguistic and cultural scenario in our country contributing one-fifth population of the world, the number proposed by ICMR appears to be arbitrary and illogical.

Given the significant difference in international limits that are unrelated to population size,[32] and that donor anonymity is being controversial issue in many countries, it is recommended that a new, internationally recognized model for calculating sperm donor limits be developed.

Consent for Artificial Insemination with Donor Semen as per ICMR Guideline 4.3

We,_____
and _____, being husband and wife and both of legal age, authorize Dr_____ to inseminate the wife artificially with semen of a donor (registration no._____; obtained from _____ semen bank) for achieving conception.

We understand that even though the insemination may be repeated as often as recommended by the doctor, there is no guarantee or assurance that pregnancy or a live birth will result.

We have also been told that the outcome of pregnancy may not be the same as those of the general pregnant population, for example in respect of abortion, multiple pregnancies, anomalies or complications of pregnancy or delivery.

We declare that we shall not attempt to find out the identity of the donor.

I, the husband, also declare that should my wife bear any child or children as a result of such insemination (s), such child or children shall be as my own and shall be my legal heir (s).

The procedure(s) carried out does (do) not ensure a positive result, nor do they guarantee a mentally and physically normal body. This consent holds good for all the cycles performed at the clinic.

Endorsement by the ART Clinic

I/we have personally explained to _____and _____ the details and implications of his/her/their signing this consent/approval form, and made sure to the extent humanly possible that he/she/they understand these details and implications.

Name, Address and Signature of the Witness from the clinic

Signed:_____
(Husband)
(Wife) _____
Name and Signature of the Doctor
Dated:

REFERENCES

1. Mortimer D. The future of male infertility management and assisted reproduction technology. Hum Reprod 2000;5:98-110.

2. Seibel MM. Therapeutic donor insemination. In: Seibel MM, Crockin Sl, eds. Family building through egg and sperm donation. London: Jones and Bartlett 1996:33-52.
3. Palermo GD, Jons H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte Lancet 1992;340:17-18.
4. Oehninger S, Veeck L, Lanzendorf S, et al. Intracytoplasmic sperm injection: Achievement of high pregnancy rates in couples with severe male factor infertility is dependent primarily upon female and not male factors Feml Stenl 1995;64:977-81.
5. Palermo GD, Cohen J, Ahkani M, et al. Intracytoplasmic sperm injection, a novel treatment for all forms of male factor infertility Feml Stenl 1995;63:1231-40.
6. Shenns RJ, Thorsell LP, Dorfmann A, et al. Intracytoplasmic sperm injection facilitates fertilization even in the most severe forms of male infertility: Pregnancy outcome correlates with maternal age and number of eggs available. Feml Stenl 1995;64:369-75.
7. Silber SJ. What forms of male infertility are there left to cure? Hum.Reprod 1995;10:503-04.
8. Braverman AJA, Corson SL. Factors related to preferences in gamete donor sources. Feml. Stenl 1995;63:543-49.
9. Rojo-Moreno J, Valdemoro C, Garcia-Merita ML, Tortajada M. Analysis of the attitudes and emotional processes in couples undergoing artificial insemination by donor. Hum. Reprod 1996;11: 294-99.
10. Brodenck P, Walker I. Information access and donated gametes how much do we know about who wants to know? Hum. Reprod 1995;10:3338-41.
11. Cook R, Golombok S, Bish A, Murray C. Disclosure of donor insemination: parental attitudes Am J Orthopsychiatry 1995;65:549-59.
12. Schover LR, Greenhalgh LF, Richards SI, Collins RX. Psychological screening and the success of donor insemination Hum. Reprod 1994;9:176-78.
13. Liu SC, Weaver SM, Robinson J, et al. A survey of semen donor attitudes. Hum. Reprod 1995;10:234-38.
14. Kovacs GT, Clayton CE, McGowan P. (1983) The attitudes of semen donors. Clin Reprod Fertil 1983;2:73-75.
15. Pedersen B, Neilsen AF, Lauritsen JG. Psychosocial aspects of donor insemination. Sperm donors–their motivations and attitudes to artificial insemination. Acta Obstet. Gynaecol. Scand 1994;73:701-05.
16. Golombok S, Cook R. A survey of semen donation: Phase I – the view of UK licensed centres. Hum Reprod 1995;10:882-88.
17. Schäfer D, Arnemann J, Brude E, et al. Society must decide about prenatal diagnosis. Hum. Reprod 1995;10:768-71.
18. Helen Lyall, Gwyn W Gould, Iain T Cameron. Should sperm donors be paid? A survey of the attitudes of the general public. Hum Reprod 1998;13(3):771-75.
19. MorJosef S, Schenker JG. Sperm donation in Israel. Hum.Reprod 1995;10:965-67.
20. Kate M Godman, Katherine Sanders, Michael Rosenberg, Peter Burton. Potential sperm donors', recipients' and their partners' opinions towards the release of identifying information in Western Australia Hum Reprod 2006;21, 11:3022-26.
21. Clarke GN, Liu DY, Baker HWG. Recovery of human sperm motility and ability to interact with the human zona pellucida after more than 28 years of storage in liquid nitrogen. Fertil Steril 2006;86:721-22.
22. Murtori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. J Androl 2003;24:253-62.
23. Clarke GN, Liu DY, Baker HWG. Recovery of human sperm motility and ability to interact with the human zona pellucida after more than 28 years of storage in liquid nitrogen. Fertil Steril 2006;86:721-22.
24. Kliesch S, Kamischke A, Nieschlag E. Cryopreservation of human semen. In: Nieschlag E, Behre HM, eds. Andrology: Male reproductive health and dysfunction. Berlin: Springer 1997:349-55.
25. World report on ART. In: European Society of Human Reproduction and Embryology. Available at: http:// www.eshre.com/emc.asp?pageId ¼ 807. Accessed on August 21, 2007.
26. McWhinnie A. Debate on gamete donation and anonymity: Should offspring from donated gametes continue to be denied knowledge of their origins and antecedents? Hum Reprod 2001;16:807-17.
27. Curie-Cohen M. The frequency of consanguineous matings due to multiple use of donors in artificial insemination. Am J Hum Genet 1980;32:589-600.
28. de Boer A, Oosterwijk JC, Rigters-Aris CAE. Determination of a maximum number of artificial inseminations by donor children per sperm donor. Fertil Steril 1995;63:419-21.
29. Wang C, Tsai M, Lee M, Huang S, Kao C, Ho H, et al. Maximum number of live births per donor in artificial insemination. Hum Reprod 2007;22:1363-72.
30. Danks DM. Genetic considerations. In:Wood C, ed. Artificial insemination by donor. Melbourne: Brown Prior Anderson 1983:94-102.
31. National Library of Health. In: Clinical Genetics Specialist Library. Available at: http://www.library.nhs.uk. Accessed on April 4, 2007.
32. Dictionary of Genetics. In: University of Edinburgh. Available at: http:// helios.bto.ed.ac.uk/bto/glossary. Accessed on April 11, 2007.
33. Kuring K. Donor limits—considerations beyond supply and demand. Melbourne: Infertility Treatment Authority 2004.
34. Janssens MW. No reason for a reduction in the number of offspring per sperm donor because of possible transmission of autosomal dominant diseases. Hum Reprod 2003;18:669-71.

SECTION 5

Special Topics

CHAPTER 23

Environmental Factors: Effects on Semen Parameters

Jaime Mendiola, Alberto M Torres-Cantero

INTRODUCTION

Several studies have suggested that human semen quality and fecundity is declining.[1-10] However, changes in semen quality may not develop uniformly.[11,12] Geographical differences in semen quality support the idea that local factors present in some areas but not in other may be influencing declines in semen quality.[13-16] Environmental pollutants, occupational exposures and lifestyle have been explored as possible contributors to those changes.[17-19]

Endocrine Disruptor Compounds (EDCs) like some polychlorinated biphenyls (PCBs),[20,21] organochlorine compounds (pesticides)[22,23] or phthalate esters (PEs),[24] several heavy metals[25-27] and polycyclic aromatic hydrocarbons (PAHs)[28] may compromise reproductive male function.

Chemicals could adversely affect male reproductive system by, either disrupting the gonadal endocrine axis[6,29] or, the spermatogenesis process (Figure 23.1). Any of those mechanisms would result in poor semen quality.[25, 30, 31] Recent studies suggest that sperm DNA integrity may be altered by environmental exposure to some toxic chemicals.[32,33] DNA fragmentation may be an excellent marker for exposure to potential reproductive toxicants and a diagnostic tool for male infertility.[34-36]

Occupational activities involving exposure to specific chemicals or expositions to toxicants may impair male reproductive health and cause infertility in humans.[26,31,36-54]

Although there is a growing body of literature relating the effect of specific substances on semen quality, the relationship between environmental chemical exposures and male infertility is more contradictory and less well documented. Only few studies have explored differences between infertile males attending fertility clinics and controls[26,39,41,43,55-57] and findings are inconclusive. While some studies found differences between fertile and infertile males in their professional activities (such as, welding, being a white collar professional or exposure to metals), their exposure to chemicals (like solvents) or to physical agents (like electromagnetic fields or heat), other studies did not find significant differences to the same or similar exposures. In this chapter we review the current evidence on the association between the main occupational and lifestyles exposures and male infertility.

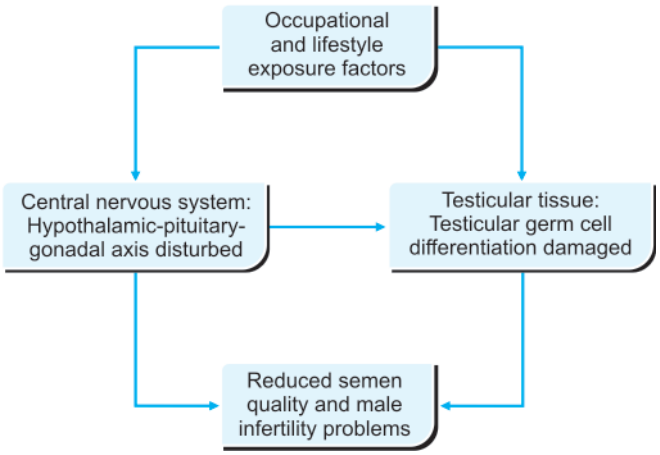


Figure 23.1: Pathways showing the relationship between occupational and lifestyle exposure factors and male infertility affectation

ENVIRONMENTAL FACTORS

Endocrine Disruptor Compounds (EDCs)

EDCs not only might cause the "testicular dysgenesis syndrome" (TDS), but also disturb meiosis in developmental germinal cells.[6, 58] Sharpe and Skakkebæk[58] have suggested that the male reproductive system is most vulnerable to estrogenic agents during the critical period of cell differentiation and organ development in fetal and neonatal life.[37] In this period, the testes are structurally organized, establishing Sertoli cell and spermatogonia numbers to support spermatogenesis that will be initiated at puberty. The maintenance of tightly regulated estrogen levels is essential for its completion.

It has also been described that these compounds cross a blood-tissue barrier similar to that of the testis, suggesting that intratubular germ cells might be exposed.[59, 60] Genistein, the principal soy isoflavone, has estrogenic activity and is widely consumed. Doerge et al (2000)[59] measured placental transfer of genistein in rats as a possible route of developmental exposure. Pregnant Sprague-Dawley rats were administered genistein orally. Concentrations of genistein aglycone and conjugates were measured in maternal and offspring serum and brain. Although fetal or neonatal serum concentrations of total genistein were approximately 20-fold lower than maternal serum concentrations, the biologically active genistein aglycone concentration was only 5-fold lower, and fetal brains contained predominately genistein aglycone at levels similar to the maternal brain. These studies show that genistein aglycone crosses the rat placenta and can reach significant levels in fetal brains.

Juhler et al[61] investigated the hypothesis that farmers that have a high intake of organic grown commodities would have a high semen quality due to their expected lower levels of dietary pesticides intake. In their conclusions authors reported that estimated dietary intake of 40 groups of pesticides did not seem to entail a risk of impaired semen quality, but as they recommended, cautions should be taken in trying to generalize these negative results to populations with higher dietary exposure levels or intakes of different groups of pesticides.

Endogenous hormones have a vital role in fetal life and ensure future fertility. Exposure to the wrong hormones (male fetus exposed to female hormones) or inadequate amounts of these, could affect the reproductive system and genitalia may not develop correctly resulting in fertility problems in adulthood.[62, 63]

Recently, Aksglaede et al[64] published a review about the sensitivity of the child to sex steroids and the possible impact of exogenous estrogens. Some of the conclusions were that children are extremely sensitive to estradiol and may respond with increased growth and/or breast development even at serum levels below the current detection limits, and that those changes in hormone levels during fetal and prepubertal development may have severe effects in adult life. The authors concluded that a cautionary approach should be taken in order to avoid unnecessary exposure of fetuses and children to exogenous sex steroids and endocrine disruptors, even at very low levels. That caution includes food intake, as possible adverse effects on human health may be expected by consumption of meat from hormone-treated animals.[65]

A recent study carried out by Swan et al[66] suggests that maternal beef consumption, and possibly xenobiotics (anabolic steroids) in beef may alter testicular development *in uterus* and adversely affect reproductive capacity. Sperm concentration was inversely related to mother's weekly beef intake. In sons of "high consumers" (>7 beef meals/week), sperm concentration was 24.3% lower than that of men whose mothers ate less beef.[66, 67]

General population is exposed to many potential endocrine disruptors concurrently. Studies, both *in vivo* and *in vitro*, have shown that the action of estrogenic compounds is additive,[68, 69] but little is known about the possible synergistic or additive effects of these compounds in humans.[70]

A recent work published by the Nordic Cryptorchidism Study Group, studied the human association between maternal exposure to 27 groups of pesticides and cryptorchidism among male children. In a nested case-control study within a prospective birth cohort, researchers compared 62 milk samples from mothers of cryptorchid boys and 68 from mothers of healthy ones and no significant differences were found for any individual chemical. However, combined statistical analysis of the eight most abundant and persistent pesticides showed that pesticide levels in breast milk were significantly higher in boys with cryptorchidism.[71]

Consequently, it is being speculated that male reproductive anomalies (hypospadias, cryptorchidism)[72] and the global fall in sperm counts[1] have both a causal link in the marked increased of phytoestrogens in our diet brought about by the western adoption of a fast food culture.[73]

Heavy Metals

Exposure to metals (mainly lead and cadmium) has been long associated with low sperm motility and density, increased morphological anomalies and male infertility.[28,74] Males employed in metal industries had a decreased fertility when compared with other workers as shown by a delayed pregnancy and reduced semen quality[25,30,39,75-81] Akinloye et al[80] analyzed the serum and seminal plasma concentrations of cadmium (Cd) in 60 infertile males and 40 normozoospermic subjects. Seminal plasma levels of Cd were significantly higher than serum levels in all subjects ($p<0.001$). A statistically significant inverse correlation was observed between serum Cd levels and all biophysical semen parameters except sperm volume. The results of that study support previous findings concerning cadmium toxicity and male infertility.

Naha et al[81] studied the blood and semen lead level concentration among battery and paint factory workers. Their results included oligozoospermia, concomitant lowering of sperm protein and nucleic acid content and increased percentage of sperm DNA haploids ($P < 0.001$), suggesting a diminution of sperm cell production after occupational lead exposure. Additionally, there was a decreased sperm velocity, reduced gross and forward progressive motility with high stationary motile spermatozoa ($P < 0.001$) suggesting retarded sperm activity among the exposed workers. Finally, they also found increased incidence of teratozoospermia associated with high blood and semen lead levels ($P < 0.001$). Other reports also have shown a significantly negative correlation between blood lead concentration and semen quality.[27,82,83]

Telisman et al[83] measured in 149 healthy industrial workers aged between 20 and 43 years semen quality and reproductive endocrine function. The group included 98 subjects with light to moderate occupational exposure to lead (Pb) and 51 with no occupational exposure. The overall study results indicated that even moderate exposures to Pb (Blood Pb < 400 µg/L) and cadmium (Cd) (Blood Cd < 10 µg/L) significantly reduced human semen quality without conclusive evidence of a parallel impairment of the male reproductive endocrine function.

Recently, Telisman et al[84] reported reproductive toxicity of low-level lead exposure in men with no occupational exposure to metals. In this study semen quality, seminal plasma indicators of secretory function of the prostate and seminal vesicles, sex hormones in serum, and biomarkers of lead, cadmium, copper, zinc, and selenium body burden were measured in 240 Croatian men 19-52 years of age. After adjustment in multiple regression a significant associations was found between blood lead (BPb) and reproductive parameters, such as immature sperm concentration, percentage of pathologic sperm, wide sperm, round and short sperm, serum levels of testosterone and estradiol and a decrease in seminal plasma zinc and in serum prolactin ($P<0.05$). These reproductive effects were observed at low-level lead exposures (median BPb 49 µg/L, range 11-149 µg/L in the 240 subjects) that are similar to those of the general population worldwide. Other articles, however, are less conclusive and show no apparent adverse effects of lead or cadmium exposure on semen quality or decreased fertility.[85-87]

Air Pollutants

Semen quality in workers exposed occupationally to hydrocarbons like toluene, benzene and xylene present anomalies, including alterations in viscosity, liquefaction capacity, sperm count, sperm motility and the proportion of sperm with normal morphology compared with unexposed males.[88-90] Similarly, exposure to solvents may affect human seminal quality[18, 51] proportionally with the range of exposure.[53] Several works have shown an association between episodic air pollution and affectation of the molecular (DNA fragmentation) and WHO seminal parameters in semen samples of young men.[91, 92]

In conclusion, there is an increasing awareness in the general population, as well as preliminary evidence, about the effect of simultaneous exposure to compounds such as food additives, toxicants, contaminants, outdoor and indoor air pollutants, endocrine disruptors and hazardous substances on semen quality and male infertility. Therefore, it is crucial to develop toxicity studies able to address complex mixtures of chemicals, both for hazard identification as well as for risk assessment of these chemical mixtures at present. Ideally, a prospective design would be more suitable to assess the effect of possible exposures along each stage of the patient's lifespan.

REFERENCES

1. Carlsen E, Giwercman A, Keiding N, et al. Evidence for decreasing quality of semen during the past 50 years. BMJ 1992;305:609-13.
2. Irvine S, Cawood E, Richardson D, et al. Evidence of deteriorating semen quality in the United Kingdom: Birth cohort study in 577 men in Scotland over 11 years. BMJ 1996;312:467-71.
3. Swan SH, Elkin EP, Fenster L. Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect 1997; 105:1228-32.
4. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: An analysis of 101 studies published 1934-1996. Environ Health Perspect 2000;108:961-66.
5. Aitken RJ, Koopman P, Lewis SE. Seeds of concern. Nature 2004; 432:48-52.
6. Skakkebæk NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod 2001;16:972-78.
7. Skakkebæk NE, Jørgensen N, Main KM, et al. Is human fecundity declining? Int J Androl 2006;29:2-11.
8. Jørgensen N, Asklund C, Carlsen E, et al. Coordinated European investigations of semen quality: Results from studies of Scandinavian young men is a matter of concern. Int J Androl 2006;29:54-61.
9. Hauser R. The environment and male fertility: Recent research on emerging chemicals and semen quality. Semin Reprod Med 2006; 24:156-67.
10. Swan SH. Does our environment affect our fertility? Some examples to help reframe the question. Semin Reprod Med 2006;24:142-46.
11. Fisch H, Goluboff ET, Olson JH, et al. Semen analyses in 1, 283 men from the United States over a 25-year period: No decline in quality. Fertil Steril 1996;65:1009-14.
12. Paulsen CA, Berman NG, Wang C. Data from men in greater Seattle area reveals no downward trend in semen quality: Further evidence that deterioration of semen quality is not geographically uniform. Fertil Steril 1996;65:1015-20.
13. Fisch H, Goluboff ET. Geographic variations in sperm counts: a potential cause of bias in studies of semen quality. Fertil Steril 1996; 65:1044-46.
14. Fisch H, Ikeguchi EF, Goluboff ET. Worldwide variations in sperm counts. Urology 1996;48:909-11.
15. Jørgensen N, Andersen AG, Eustache F, et al. Regional differences in semen quality in Europe. Hum Reprod 2001;16:1012-19.
16. Swan SH, Brazil C, Drobnis EZ, et al. Geographic differences in semen quality of fertile US males. Environ Health Perspect 2003; 111:414-20.
17. Figà-Talamanca I, Cini C, Varricchio GC. Effects of prolonged autovehicle driving on male reproduction function: A study among taxi drivers. Am J Ind Med 1996;30:750-58.
18. Tielemans E, Burdorf A, te Velde ER, et al. Occupationally related exposures and reduced semen quality: A case-control study. Fertil Steril 1999;71:690-96.
19. Homan GF, Davies M, Norman R. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review. Hum Reprod Update 2007;13:209-23.
20. Rozati R, Reddy PP, Reddanna P, et al. Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 2002;78:1187-94.
21. Spanò M, Toft G, Hagmar L, et al. Exposure to PCB and p,p´-DDE in European and Inuit populations: Impact on human sperm chromatin integrity. Hum Reprod 2005;20:3488-99.
22. Swan SH. Semen quality in fertile US men in relation to geographical area and pesticide exposure. Int J Androl 2005;29:62-68.
23. Carreño J, Rivas A, Granada A, et al. Exposure of young men to organochlorine pesticides in Southern Spain. Environ Res 2007; 103:55-61.
24. Duty SM, Silva MJ, Barr DB, et al. Phthalate exposure and human semen parameters. Epidemiology 2003;14:269-77.
25. Robins TG, Bornman MS, Ehrlich RI, et al. Semen quality and fertility of men employed in a South African lead acid battery plant. Am J Ind Med 1997;32:369-76.
26. Irgens A, Krüger K, Ulstein M. The effect of male occupational exposure in infertile couples in Norway. J Occup Environ Med 1999; 41:1116-20.
27. Benoff S, Jacob A, Hurley IR. Male infertility and environmental exposure to lead and cadmium. Hum Reprod Update 2000;6:107-21.
28. Wagner U, Schlebusch H, Van der Ven H, et al. Accumulation of pollutants in the genital tract of sterility patients. J Clin Chem Clin Biochem 1990;28:683-88.
29. Sharpe RM, Irvine DS. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? BMJ 2004;328:447-51.
30. Assennato G, Paci C, Baser ME, et al. Sperm count suppression without endocrine dysfunction in lead-exposed men. Arch Environ Health 1987;42:124-27.
31. Wyrobek AJ, Schrader SM, Perreault SD, et al. Assessment of reproductive disorders and birth defects in communities near hazardous chemical sites. III Guidelines for field studies of male reproductive disorders. Reprod Toxicol 1997;11:243-59.
32. Stronati A, Manicardi GC, Cecati M, et al. Relationship between sperm DNA fragmentation, sperm apoptotic markers and serum levels of CB-153 and p,p´-DDE in European and Inuit populations. Reproduction 2006;132:949-58.
33. Aitken RJ, De Luiis GN. Origins and consequences of DNA damage in male germ cells. Reprod Biomed Online 2007;14:727-33.
34. Evenson DP, Wixon R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm

Chromatin Structure Assay (SCSA^{Ò}). Toxicol Appl Pharmacol 2005; 207:(2 Suppl), 532-37.
35. Ozmen B, Caglar GS, Koster F, et al. Relationship between sperm DNA damage, induced acrosome reaction and viability in ICSI patients. Reprod Biomed Online 2007;15:208-14.
36. Mortensen JT. Risk for reduced sperm quality among metal workers, with special reference to welders. Scand J Work Environ Health 1988; 14:27-30.
37. Ng TP, Goh HN, Ng YL, et al. Male endocrine functions in workers with moderate exposure to lead. Br J Ind Med 1991;48:485-91.
38. Lerda D. Study of sperm characteristics in sperm in persons occupationally exposed to lead. Am J Ind Med 1992;22:567-71.
39. Bonde P. The risk of male subfecundity attributable to welding of metals. Studies of semen quality, infertility, fertility, adverse pregnancy outcome and childhood malignancy. Int J Androl 1993; 16:Suppl 1, 1-29.
40. Fredricsson B, Moller L, Pousette A, et al. Human sperm motility is affected by plasticizers and diesel particle extracts. Pharmacol Toxicol 1993; 72:128-33.
41. Lähdetie J. Occupational- and exposure-related studies on human sperm. J Occup Environ Med 1995;37:922-30.
42. Alexander BH, Checkoway H, Faustman EM, et al. Contrasting associations of blood and semen lead concentrations with semen quality among lead smelter workers. Am J Ind Med 1998;34:464-69.
43. Bigelow PL, Jarrell J, Young MR, et al. Association of semen quality and occupational factors: Comparison of case-control analysis and analysis of continuous variables. Fertil Steril 1998;69:11-18.
44. Zenzes MT, Bielecki R, Reed TE. Detection of benzo(a)pyrene diolepoxide-DNA adducts in sperm of men exposed to cigarette smoke. Fertil Steril 1999;72:330-35.
45. Viskum S, Rabjerg L, Jorgensen PJ, et al. Improvement in semen quality associated with decreasing occupational lead exposure. Am J Ind Med 1999;35:257-63.
46. Sallmen M, Lindbohm ML, Nurminen M. Paternal exposure to lead and infertility. Epidemiology 2000;11:148-52.
47. Figà-Talamanca I, Traina ME, Urbani E. Occupational exposures to metals, solvents and pesticides: recent evidence on male reproductive effects and biological markers. Occup Med (Lond) 2001; 51:174-88.
48. Sheiner EK, Sheiner E, Hammel RD, et al. Effect of occupational exposures on male fertility: literature review. Ind Health 2003;41:55-62.
49. Meeker JD, Rossano MG, Protas B, Diamond MP, Puscheck E, Daly D, et al. Cadmium, Lead and Other Metals in Relation to Semen Quality: Human Evidence for Molybdenum as a Male Reproductive Toxicant. Environ Health Perspect 2009;116:1473-79.
50. Chang HY, Shih TS, Guo YL, et al. Sperm function in workers exposed to N,N-dimethylformamide in the synthetic leather industry. Fertil Steril 2004;81:1589-94.
51. Jensen TK, Bonde JP, Joffe M. The influence of occupational exposure on male reproductive function. Occup Med (Lond) 2006;56:544-53.
52. Queiroz EK, Waissmann W. Occupational exposure and effects on the male reproductive system. Cad Saude Publica 2006; 22: 485-93.
53. Cherry N, Moore H, McNamee R, et al. Occupation and male infertility: Glycol ethers and other exposures. Occup Environ Med 2008, in press
54. Mendiola J, Torres-Cantero AM, Moreno-Grau JM, et al. Exposure to environmental toxins in males seeking infertility treatment: A case-controlled study. Reprod Biomed Online 2008;16:842-50.
55. Chia SE, Lim ST, Tay SK, et al. Factors associated with male infertility: A case-control study of 218 infertile and 240 fertile men. BJOG 2000; 107:55-61.
56. Chia SE, Tay SK. Occupational risk for male infertility: A case-control study of 218 infertile men and 227 fertile men. J Occup Environ Med 2001;43:946-51.
57. Cherry N, Labrèche F, Collins J, et al. Occupational exposure to solvents and male infertility. Occup Environ Med 2001; 58:635-640.
58. Sharpe RM, Skakkebæk NE. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993; 341:1392-95.
59. Doerge DR, Churchwell MI, Chang HC, et al. Placental transfer of the soy isoflavone genistein following dietary and gavage administration to Sprague Dawley rats. Reprod Toxicol 2001;15:105-10.
60. West MC, Anderson L, McClure N, et al. Dietary oestrogens and male fertility potential. Hum Fertil 2005;8:197-207.
61. Juhler RK, Larsen SB, Meyer O, et al. Human semen quality in relation to dietary pesticide exposure and organic diet. Arch Environ Contam Toxicol 1999;37:415-23.
62. Sharpe RM. Pathways of endocrine disruption during male sexual differentiation and masculinisation. Best Pract Res Clin Endocrinol Metab 2006;20:91-110.
63. Sharpe RM, Franks S. Environment, lifestyle and infertility- an inter-generational issue. Nat Cell Biol 2002;4:s33-40.
64. Aksglaede L, Juul A, Leffers H, et al. The sensitivity of the child to sex steroids: possible empact of exogenous estrogens. Hum Reprod Update 2006;12:341-49.
65. Andersson AM, Skakkebæk NE. Exposure to exogenous estrogens in food: Possible impact on human development and health. Eur J Endocrinol 1999;140:477-85.
66. Swan SH, Liu F, Overstreet JW, et al. Semen quality of fertile US males in relation to their mothers´ beef consumption during pregnancy. Hum Reprod 2007; 22:1497-1502.
67. vom Saal FS. Could hormone residues be involved? Hum Reprod 2007;22:1503-05.

68. Rajapakse N, Silva E, Kortenkamp A. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ Health Perspect 2002;110:917-21.
69. Tinwell H, Ashby J. Sensitivity of the immature rat uterotrophic assay to mixtures of estrogens. Environ Health Perspect 2004; 112:575-82.
70. Toppari J, Skakkebæk NE. Sexual differentiation and environmental endocrine disrupters. Baillieres Clin Endocrinol Metab 1998;12:143-56.
71. Damgaard IN, Skakkebæk, Toppari J, et al. Persistent pesticides in human breast milk and Cryptorchidism. Environ Health Perspect 2006;114:1133-38.
72. Toppari J, Kaleva M, Virtanen HE. Trends in the incidence of cryptorchidism and hypospadias, and methodological limitations of registry-based data. Hum Reprod Update 2001;7:282-86.
73. O´Donnell L, Robertson KM, Jones ME, et al. Estrogens and spermatogenesis. Endocr Rev 2001;22:289-318.
74. Umeyama T, Ishikawa H, Takeshima H, et al. A comparative study of seminal plasma trace elements in fertile and infertile men. Fertil Steril 1986;46:494-99.
75. Xuezhi J, Youxin L, Yilan W. Studies of lead exposure on reproductive system: A review of work in China. Biomed Environ Sci 1992;5:266-75.
76. Gennart JP, Buchet JP, Roels H, et al. Fertility of male workers exposed to cadmium, lead or manganese. Am J Epidemiol 1992; 135:1208-19.
77. Chia SE, Chan OY, Sam CT, et al. Blood cadmium levels in non-occupationally exposed adult subjects in Singapore. Sci Total Environ 1994;145:119-23.
78. Spinelli A, Figa-Talamanca, Osborn J. Time to pregnancy and occupation in a group of Italian women. Int J Epidemiol 1997;26: 601-09.
79. Danadevi K, Rozati R, Reddy PP, et al. Semen quality of Indian welders occupationally exposed to nickel and chromium. Reprod Toxicol 2003;17:451-56.
80. Akinloye O, Arowojolu AO, Shittu OB, et al. Cadmium toxicity: A possible cause of male infertility in Nigeria. Reprod Biol 2006;6:17-30.
81. Naha N, Chowdhury AR. Inorganic lead exposure in battery and paint factory: Effect on human sperm structure and functional activity. J UOEH 2006;28:157-71.
82. Benoff S, Hurley IR, Millan C, et al. Seminal lead concentrations negatively affect outcomes of artificial insemination. Fertil Steril 2003; 80:517-25.
83. Telisman S, Cvitkovic P, Jurasovic J, et al. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc and copper in men. Environ Health Perspect 2000; 108:45-53.
84. Telisman S, Colak B, Pizent A, et al. Reproductive toxicity of low-level lead exposure in men. Environ Res 2007;105:256-66.
85. Coste J, Mandereau L, Pessione F, et al. Lead-exposed workman and fertility: A cohort study on 354 subjects. Eur J Epidemiol 1991; 7:154-58.
86. Abou-Shakra FR, Ward NI, Everard DM. The role of trace elements in male infertility. Fertil Steril 1989;52:307-10.
87. Hovatta O, Venalainen ER, Kuusimaki L, et al. Aluminium, lead and cadmium concentrations in seminal plasma and spermatozoa, and semen quality in Finnish men. Hum Reprod 1998;13:115-19.
88. Xiao G, Pan C, Cai Y, et al. Effect of benzece, toluene, xylene on the semen quality of exposed workers. Chin Med J 1999;112:709-12.
89. De Celis R, Feria-Velasco A, González-Unzaga M, et al. Semen quality of workers occupationally exposed to hydrocarbons. Fertil Steril 2000; 73:221-8.
90. De Rosa M, Zarrilli S, Paesano L, et al. Traffic pollutants affect fertility in men. Hum Reprod 2003;18:1055-61.
91. Selevan SG, Borkovec L, Slott VL, et al. Semen quality and reproductive health of young Czech men exposed to seasonal air pollution. Environ Health Perspect 2000;108:887-94.
92. Rubes J, Selevan SG, Evenson DP, et al. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod 2005;20:2776-86.

CHAPTER 24

The Aging Male

Vijay Mangoli

Getting older is part of life. We all know it and at some point, accept it. Though one can remain 'young at heart' for ever, all our senses and systems have limitations. Their efficiency is inversely proportional to age. Due to various advances in medical fields, life expectancy has doubled particularly in developed countries, and also greatly increased in developing countries.

There is no specific disease attributed to age. Furthermore, every individual responds uniquely to process of aging and to the complications arising due to treatment protocols in different pathological conditions. Aging involves series of morphological and functional modifications within organs leading to reduced efficiency and atrophy of various tissues and systems.[1] Different theories have been postulated for the process of aging. Aging can be a genetically fixed program, it may be due to spontaneous mutations that affect the regeneration and repair mechanism, it may be due to excessive accumulation of waste products like lipofuscin and free oxygen radicals, or it may be due to production of abnormal immunological products, which are not recognized by immunocompetent cells for proper disposal, hence causing irreparable damages.

It is believed and proven that men retain their ability to father a child till the age as old as 95 years. Men do not go through a traditional endocrinological menopause. Until recently we had a poor understanding of the effect of aging on male fertility. As many examples have been offered of older men having babies, the thought of decreased fertility in men was never greatly addressed. It was assumed that male fertility was relatively immortal because so many elderly men have been able to impregnate their wives. However, there has been published data showing a relative decrease in sperm count, and possibly fertility, in a certain percentage of aging men. Now, the field of male fertility has become mainstream and full of new research, new data and new conclusions.

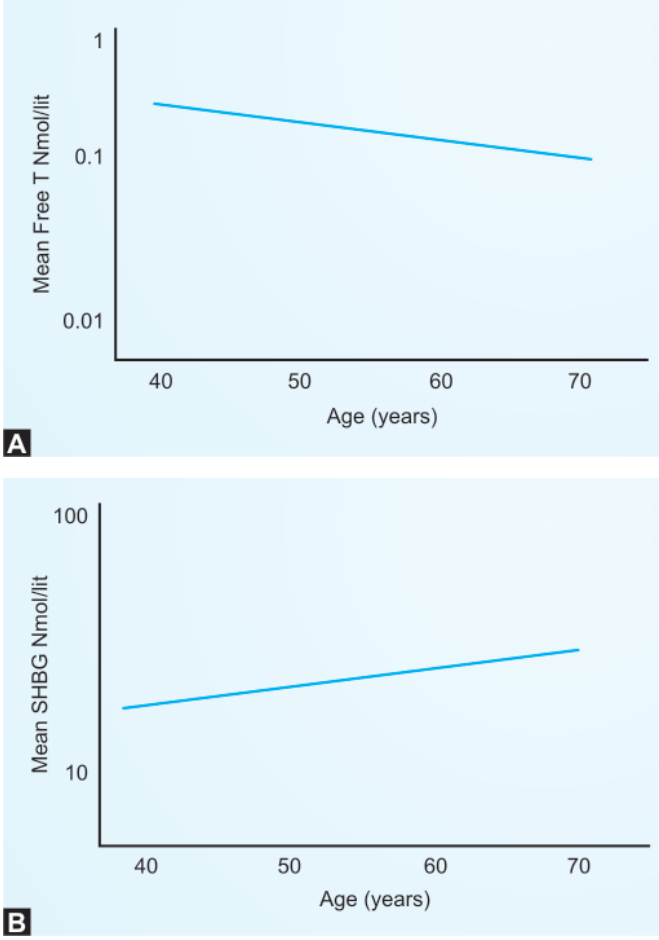
Physiology and Sexual Differentiation

Aging also involves major endocrinological changes. Growth hormone (GH) production is age dependent. It reaches its peak at puberty and then decreases continuously, resulting in reduced muscle strength and increased BMI.[2]

In case of fertility, it is widely known that female gradually becomes less fertile after the age of 35, and eventually undergoes menopause between the ages of 45 to 55 years. The consequences of endocrine changes in the female have been investigated for many years. In contrast, the knowledge about male endocrine changes and its effect on aging lags far behind. There is no obvious or sudden change in reproductive behavior or loss of fertility in men comparable to menopause. The testosterone levels and testicular volumes of aging men without accompanying diseases are quite comparable to younger men. However, a reduced endocrine reserve capacity of the testicular Leydig cells and the pituitary gonadotrophs is observed. Younger men's response to hCG in increasing testosterone levels is much higher than in aging men. Similarly, increase in luteinizing hormone (LH) as a response to Gonadotropin Releasing Hormone (GnRH) is much higher in younger men.[3]

Testosterone levels in serum of older men was found to be lower than young population but with considerable variance. Nieschlag reported that most of the testosterone in serum is protein bound. In older men, the proportion of sex hormone binding globulin (SHBG) in serum increases and hence the proportion of bioactive free testosterone decreases[4] (Figure 24.1).

Lower testosterone levels and higher sex hormone binding globulin can intensify estrogen effect of an individual, thus causing increased incidences of gynecomastia in older men.[5] Male development in mammals largely depends upon the inheritance of a Y chromosome.[6] The Y chromosome is largely heterochromatic and contains nucleotide sequence that are repeated many times and that do not seem to affect sexual differentiation.[7] Primordial germ cells differentiate very early in embryos. The human gonad differentiates as ovary or testis between days 36 and 42 in fetuses. Male primordial germ cells proliferate between 2-6 months, and then differentiate into prospermatogonia. This is the first wave of rapid proliferation. The second wave in the testis begins at puberty and continues throughout life.[8] The testis descends during later fetal growth through the inguinal canal. The adult testes contain seminiferous tubules that have differentiated from rete cords. Human has more than 500 tubules. As men age, regressive vascular changes in the tubular wall are associated with increasing fibrosis and hyalinosis.[9] Spermatogenesis and spermiogenesis are established and sustained from puberty by FSH and LH. The testes are highly active endocrinologically, producing steroids and other hormones from Leydig and Sertoli cells.[10] Aging in male is known for disorders in either central endocrine regulation or the peripheral control of spermatogenesis and spermiogenesis. The secondary spermatocyte differentiate into a spermatid and then into a spermatozoon during spermiogenesis. Spermatogonal multiplication might be regulated by Sertoli cells or by feedback actions between proliferating spermatogonia, and it declines in many men aged 60 and above.[11] The number of sperm cells produced is the main focus of studies in aging men and reduction in quantity is assumed to be primary cause of sub-fertility. The age-related decline in daily sperm production results largely from a block to further produce sperm that can and do mature in the early prophase stage of production. In other words, there is no difference between older men and younger men in the number of early primary sperm cells per gram of testicular tissue. However, there is a vast difference between older and younger men in the number of late (or mature) sperm cells.



Figures 24.1A and B: Free testosterone and SHBG levels in healthy men of different age groups (*Courtesy:* Gray et al 1991)

As testes from normal, healthy men of both young and of older age are extremely difficult and not justifiable for biopsies, proper study of comparison between the two groups becomes difficult. Almost all such studies have come from corpse resulting out of accidents. Other studies include diseased men mainly having prostatic carcinoma. Handelsman and Staraj reported no major difference in testicular volume of young and old.[12]

In contrast, some groups have reported that men may experience decease in size and volume of testes with advanced age. Decrease in testosterone level in aging men may contribute to decrease in bone and muscle mass in the aging male, the loss of sex-drive, the decreased ability of the body to produce and mature sperm cells, as well as the inability to obtain or maintain an erection. Both

the decrease in testosterone and the decrease in sperm production cause an age-related decrease in fertility. Though it is believed that sperm production may also be affected by repeated ejaculation decreasing the secretions of the glands, the decrease of the number of hormones and the weakening of the sexual muscles, recent studies show that frequent ejaculations help in preventing ejaculatory dysfunction (ED).

According to American infertility association, age can influence following factors causing infertility:

1. *Motility*—due to more number of immature sperm in the ejaculate, the average motility decreases therefore less number of sperm can reach fertilization site. Furthermore, the ejaculatory force also decreases due to weak bulbocavernosus muscular contractions during orgasm causing majority of sperm fraction to remain near vagina.
2. *Penetration*—immature sperm have poor penetration ability required to invade through zona pellucida.
3. *Altered genetic makeup*—advanced age is associated with increased mutations which are passed on to next generation. As sperm continues process of division, each successive division introduces a slight risk of error in the genetic material of the new sperm, which is passed on to the children.

Sperm studies in aging men often show kinetic disorders particularly reproduction, dividing and development of the sperm cell or spermatid (a maturing sperm cell) malformations, paralleled by a highly significant decrease in daily sperm production. New research indicates that the genetic quality of sperm worsens as men get older, increasing a man's risk of being infertile, fathering unsuccessful pregnancies and passing along dwarfism and possibly other genetic diseases to his children.

A study led by Andrew Wyrobek at Lawrence Livermore National Laboratory (LLNL) and the University of California, Berkeley, found a steady increase in sperm DNA fragmentation with increasing age of the study participants, along with increases in a gene mutation that causes achondroplasia, or dwarfism (Figure 24.2). Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies (chromosome abnormalities) in sperm, according to the online edition of the *Proceedings of the National Academy of Sciences.*

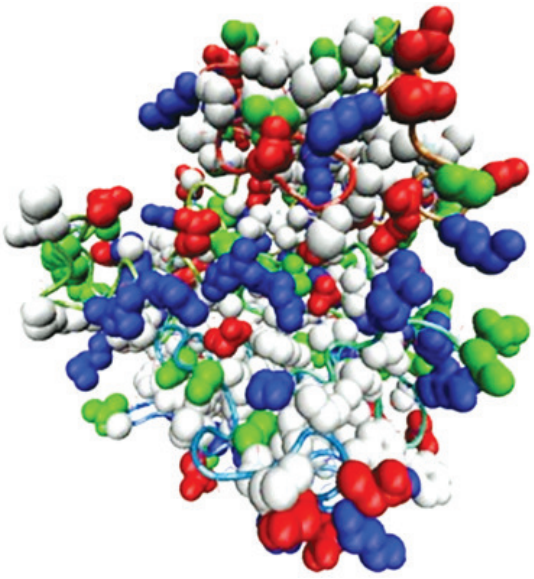


Figure 24.2: Mutations in the fibroblast growth factor receptor 3 (FGFR 3) gene (above) have been linked to achondroplasia, or dwarfism. Sperm analysis shows that mutations associated with dwarfism gradually increased by about two percent for every year of age (*Courtesy:* Andrew Wyrobek)

Wyrobek, Eskenazi and their colleagues analyzed semen from the volunteers using a variety of state-of-the art methods for detecting genetic and chromosomal defects in human sperm. A flow cytometer method was used to detect DNA fragmentation and chromatin defects in collaboration with co-author Don Evenson at South Dakota State University. Gene mutations in the achrondroplasia gene and in the Apert sydrome gene were measured using highly sensitive PCR-based methods developed by co-authors Ethylin Jabs at Johns Hopkins and Norman Arnheim at USC in Los Angeles. The team also used a Livermore-developed chromosome analysis system called sperm FISH (fluorescence *in situ* hybridization). The study included at least 15 men from each age decade from 20 to 60 years, and 25 men 60 to 80 years old. The researchers gathered extensive medical, lifestyle and occupational exposure history from the men and excluded current cigarette smokers and men with current fertility or reproductive problems, a previous semen analysis with zero sperm count, vasectomy, history of prostate cancer or undescended testicle, or exposure to chemotherapy or radiation treatment for cancer.

They found a strong correlation between age and sperm DNA fragmentation, with genetic mutations

associated with dwarfism gradually increasing by about two percent for every year of age. This study indicates that men who wait till their late 40's to have children, not only increase risk in miscarriages, they also increase probability of passing on genetic abnormalities to the child.

Though a pattern of chromosomal alteration was found in aging men, there was no clear indication that age increase the probability of Down's syndrome, Klinefelter syndrome, Turner syndrome or other forms of trisomies like triple X syndrome, and XYY in offspring– that are associated with varying types and severity of infertility as well as physical and neurological abnormalities.

Estrogens are known to be the essential sex steroid acting on some male physiological functions. Bioavailable estrogens comprise the free and albumin-bound fractions. Moreover, most of the bioavailable sex steroid is made up of albumin-bound fraction. When the age-related change in serum free, albumin-bound and bioavailable estradiol levels in comparison with each fraction of testosterone and its relationship with serum albumin level in elderly men were examined, albumin-bound as well as bioavailable estradiol levels were found to get declined with age, and their decreases were associated much more with the decrease of albumin level than the increase of sex-hormone binding globulin (SHBG) level in sixties and seventies. Similar results were obtained in the level of each fraction of testosterone, suggesting albumin levels have an important role for maintaining bioavailable sex steroid levels in males aged over sixty. SHBG levels associated inversely with bioavailable sex steroid levels particularly when serum albumin level was low. It seems likely that the decrease of bioavailable estradiol as well as testosterone is induced by the decrease of albumin-bound fractions in combination with the increase of SHBG-bound fractions in males aged over sixty, and that their physical characteristics of aging could be induced by the decrease of albumin-bound fractions caused by the decrease of serum albumin regardless of total sex steroid levels.[13]

It is known that declining endocrine capacity of testes is age related phenomena; however, an effective treatment to restore serum hormone levels in aging male is not standardized yet. In contrast, an effective hormone replacement therapy is widely applied in postmenopausal females, resulting in dramatic improvement in their lifestyle.

Hypogonadism in male, i.e. reduced testicular function is characterized by lack of energy, depression, erectile dysfunction, anemia, reduced muscle mass, obesity and osteoporosis. These are due to androgen deficiency, the incidence of which increases with advancing age. If no other obvious cause like testicular carcinoma is diagnosed for testosterone decrease, T substitution therapy can be an effective alternative. Regardless of age, serum T levels below 12 nmol/lit along with androgen deficiency symptoms can be regarded as indication for T substitution. While long acting injectable T esters are effective in younger men, shorter acting preparations are more useful in elderly men.[14]

AGING MEN AND ART

In assisted reproductive technology, particularly with Intra-cytoplasmic Sperm Injection technique, one can select at least morphologically normal looking spermatozoa for fertilization. Hence, though there are some reports of increase in sperm abnormality in aging men, it can be taken care of. However, it is not possible to avoid sperm with chromosomal abnormality by merely observing under inverted microscope. This fact has adverse implications even in younger male undergoing IVF for male factor indication. The situation may arise that a morphologically normal but chromosomally abnormal sperm is selected amongst complete healthy sperms.

Another factor that decides fertilization outcome is oocyte quality. Generally, sperms of elderly man are used to fertilize oocytes of his partner, who is also most likely an aging woman. Therefore, increased incidences of chromosomal abnormalities in resulting embryos may be attributed to either male, or female or both.

Recently, in ESHRE meeting of 2008, Dr Stéphanie Belloc, of the Eylau Centre for Assisted Reproduction, Paris, France, presented a data comparing Intrauterine Insemination outcome between the groups where men were less than 40 years and those over 40 years. The study included 21239 IUI cycles from 12236 couples. The sperm of each partner was examined at the time of the IUI for a number of characteristics, including sperm count, motility and morphology. Clinical pregnancy, miscarriage and delivery rates were also carefully recorded. Detailed analysis of the results allowed the scientists to separate out the male and female factors related to each pregnancy. Dr Belloc concluded that there is a definite increase in

miscarriage rate in the group of men over 40 years. The team also suggested IVF or ICSI as an effective option for elderly couples who want to start their family late. ICSI is more effective because a morphologically healthy sperm can be selected to fertilize the oocyte. They assumed the increased miscarriage rate may be due to increased DNA damage in more number of sperms in aging men. However, there is no study done on *in vitro* fertilization where, sperms of elderly male were used for younger female partner to evaluate pregnancy outcome or fertilization, cleavage rate associated with chromosomal abnormality.

The effect of aging in men on fertility is a complicated issue to study due to many other illnesses associated with age that affects fertility like diabetes, heart problems, blood pressure, endocrinological disorders, etc. More attention is now being given to factors involved in male fertility due to age factor apart from other common causes like oligoasthenoteratozoospermia, obstructive, non-obstructive azoospermia, CBAVD. Increased incidences of abortions and genetic abnormalities in newborn from elderly fathers demands proper counseling and awareness drive in such couples before offering fertility treatment.

REFERENCES

1. DeNicola P. Geriatrics. A Textbook. Shewer, Stuttgart.
2. de Boer H, Blok G J, van der veen EA. Clinical aspects of growth hormone deficiency in adults. Endocr. Rev 1995;16:63-86.
3. Nieschlag E, Lammers U, Freischem CW, Langer K, Wickings EJ. Reproductive functions in young fathers and grand fathers. J Clin Endocrinol Metab 1982;55:676-681 (Vermeulen A. Environment, human reproduction, menopause and andropause. Environ Health Perspect. 1993;Supple 2:91-100.)
4. Gray A, Feldmen HA, McKinlay JB, Longcope C. Age and changing sex hormone levels in middle aged men: results of the Massachusetts male aging study. J Clin Endorinol Metab 1991;73: 1016-25.
5. Morley JE, Kaiser PE, Johnson LE. Male sexual function. In: Cassel CK, Riesenberg DE, Sorensen LB, Walsh JR (Eds) Geriatric medicine, 2nd (edn), Springer Berlin Heidelberg NewYork 1990;256-70.
6. Ferguson-Smith MA; J Med Genet 1965;2:142-45.
7. Cooke H, Fantes J, Gren D. Differentiation 1983:23 (suppl 1):48-55.
8. Hilscher W. Hum Reprod; 1991; 6:1416-25). (Hilscher B, Engemann A. histological and morphometric studies on kinetics of germ cells and immature sertoli cells during human spermatogenesis. Werner-Hilscher symposium on Keimbahn and male fertility, 1991.
9. Vilar O. Spermatogenesis. In Hafez ESE, Evans TN (Eds) Human Reproduction. Hagerstown, MD Harper and Row 1973:39-51.
10. Midgley AR, Jr Gay VL, Keyes PL, Hunter JS. Human reproductive endocrinology. In Hafez ESE Evans TN (Eds). Hagerstown, MD Harper & Row, 1973:201-36.
11. De Rooji DG. J. Cell. Science 1989;(suppl) 10:181.
12. Handelsman DJ, Staraj S. Testicular size: The effects of aging, malnutrition, and illness. J Androl 1985;6:144-51.
13. Tetsuo Hayashi, Takumi Yamada. Association of bioavailable estradiol levels and testosterone levels with serum albumin levels in elderly men. The Aging Male, Volume 11, Issue 2 June 2008; pages 63-70
14. Nieschlag E, Rolf C, Zitzmann M. Abstracts of 17th annual meeting of ESHRE, 2001.

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