

# HANDBOOK OF ANDROLOGY

**Second Edition**



**The American Society of Andrology**

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## Preface

Since the first edition of the Handbook of Andrology, published by the American Society of Andrology 15 years ago, over 20,000 printed copies, as well as uncounted numbers of electronic copies, have been distributed to colleagues and trainees around the world. The first edition was translated into Spanish, Italian and Chinese. Most of the fundamental information provided in the first edition is still valid; however, the volume of scientific literature on the various facets of the subject of andrology has expanded tremendously. This has resulted in a constantly growing body of knowledge, not only in basic science but also in the clinical management of men's health issues.

In this edition, we have increased the number of chapters from 24 to 41, but have made every effort to retain a style that will allow trainees to be introduced to the field of Andrology and become as excited about working in this field as are the contributing authors. Our chapters encompass the wide range of topics that characterizes the field of Andrology, from molecular biology to veterinary and human medicine, from applied research to ethics. We once again have been extremely fortunate to have as authors world-renowned Andrologists who are experts in the various subjects included in the Handbook; we wish to thank each of them for their valuable contributions.

With the advances in information technology, we feel that it is time to drop the traditional format of a paper bound book and move to an electronic only version for this edition of the Handbook. With the support of the American Society of Andrology, this edition of the Handbook will be freely available to all members of the Society. The electronic version is available as a single PDF or as PDFs of individual chapters. We believe that this approach will allow for a wider scope of circulation of the Handbook, permit more frequent updates of the content, and help save a forest of trees.

It is our hope that this second edition of the Handbook of Andrology lays the foundation for basic scientists, clinician scientists, healthcare professionals, trainees, policy makers and anyone who has an interest in the discipline to acquire the relevant knowledge that they seek.

Finally, we would like to acknowledge the dedicated secretarial support of Ms. Elise Boivin-Ford and to thank all the contributors and various members of the American Society of Andrology for their assistance and support in making this Handbook possible.

Bernard Robaire and Peter Chan  
Co-Editors  
February 2010

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## Forward by Philip Troen

Reprinted from the first edition of the Handbook of Andrology

### “What a piece of work is a man”

Shakespeare, *Hamlet*, Act ii, scene 2, line 316

Why a handbook of andrology? Some handbooks are published to bring together multiple aspects of a diversified subject. Some handbooks are designed to present an overview of a rapidly expanding subject for those working in the field while other handbooks are intended to codify the progress already made. Although there are elements of each of these approaches in this handbook of andrology, our main purpose is to present to scientists/clinicians early in their careers the scope, importance, and excitement of our discipline.

What is andrology? Simplistically, one might say that andrology is to the male what gynecology is to the female. That is, andrology deals with matters affecting the male reproductive system. The earliest use of the term andrology, as reported by Mikko Niemi, appears to have been in 1891 in the Journal of the American Medical Association, reporting on the formation of the American Andrological Association. Little more was heard from that association and it was not until the latter half of this century that there emerged an andrology journal in 1969 and an active andrology organization, Comit  Internaci n de Andrologia, in 1970. In the quarter century since, there has been a veritable explosion of journals and publications, of societies and congresses, and of workshops and symposia devoted to andrology. Thus, we appear to be on a rapidly rising growth curve of knowledge and application in andrology. The scope of modern day andrology is strikingly indicated by the range of topics discussed in this handbook. Written by distinguished leaders in their fields, these topics were chosen to indicate the exciting breadth of andrology and the opportunity it holds for graduate students in the biological sciences, veterinary students, and medical students.

Why become an andrologist? In choosing a career one, first of all, tries to identify an area of interest. Then one looks at the opportunity to succeed and the likelihood of making a contribution. As an andrologist for 40 years, I can testify to the continued excitement and interest the discipline has held for me. As noted above, there has been a rapid escalation of growth so that our discipline now ranges from genetic studies to pubertal changes in the male and from infertility and assisted reproduction techniques to disorders of the

prostate, sexual function and contraception. Advances in these and other areas have been made possible by a remarkable series of clinical studies and scientific discoveries using the classical disciplines of physiology, biochemistry, neuroscience, and molecular biology. As we have entered each new stage of understanding and science, there has been no waning of the stimulus that I and my colleagues experience. At the same time, because of the multidisciplinary nature of andrology, unsolved problems present themselves and the opportunities for advancement and success continue to expand. As Alexander Albert has noted, "Nature has experimented lavishly with the reproductive system." This fact provides both challenge and opportunity. Andrology covers a wide spectrum from before conception to aging. As you peruse this handbook, we hope you will appreciate the scope of the field and share our excitement in the study of andrology.

# Handbook of Andrology

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## What are the components of the male reproductive system?

*CNS, pituitary, testis, epididymis, prostate,  
seminal vesicles, scrotum, penis*

K.P. Roberts

The male reproductive system consists of several organs acting together to produce functional spermatozoa, and to deliver these spermatozoa to the female reproductive tract. The system is summarized in Figure 1. Spermatozoa, the haploid germ cells, are produced in the testis and undergo maturational changes as they transit the epididymis. The vas deferens transports the spermatozoa from the epididymis to the ejaculatory duct in the prostate. The spermatozoa and secretions of the seminal vesicles empty together, with secretions from the prostate, into the prostatic urethra. Secretions from the bulbourethral gland contribute to the ejaculate as the mixture exits the body through the penile urethra. The entire system is dependent on testosterone, also produced in the testis, and its regulation by the pituitary and hypothalamus. Knowledge of the anatomy and embryological origins of the components of the male reproductive tract is important in developing a basic and thorough understanding of the system as a whole.

### Testis

The testis is the site of germ cell development (spermatogenesis) and thus the primary tissue of the male reproductive system. It is also the site of androgen synthesis and secretion. Spermatogenesis occurs within the seminiferous tubules of the testis; the process whereby germ cells progress from haploid cells (spermatogonia) to meiotic cells (spermatocytes) to haploid cells (spermatids). The epithelial cells of the seminiferous tubules, the Sertoli cells, facilitate this process. Leydig cells, that populate the interstitial space between the seminiferous tubules, produce testosterone.

The testis arises from the primitive gonad in which primitive germ cells migrate to the undifferentiated gonad from the yolk sac, causing the coelomic epithelial cells to proliferate and form the sex cords. Formation of the sex cords gives this region a raised contour that is called the genital ridge. During the fourth month of embryogenesis, the sex cords become U-shaped and their ends anastomose to form the rete testis. At this point, the primordial sex cells are referred to as pre-spermatogonia and the epithelial cells of the sex cords as Sertoli cells. The sex cords will become the seminiferous tubules.

The rete testis extends into the mesonephric tissue and will anastomose with some of the mesonephric tubules forming the efferent ducts that communicate with the epididymis (discussed below). The mesenchymal tissue in the interstitial space between the tubules gives rise to the Leydig cells, the site of androgen production.

### Scrotum

The function of the scrotum is to house and protect the testis, and to help maintain the temperature of the testis optimal for spermatogenesis. The testis develops on the posterior abdominal wall and descends into the scrotum late in development. Successful descent of the testis into the scrotum is essential for fertility. The scrotum is formed as the coelomic epithelium penetrates the abdominal wall and protrudes into the genital swelling as the processus vaginalis. An outgrowth of most layers of the abdominal wall is carried with this epithelium, giving rise to the fascial layers of the scrotum. The testes then descend behind the processus vaginalis into the scrotum. The cremasteric and Dartos layers of scrotal fascia contribute to the important temperature regulatory function of the scrotum.

### Epididymis

The epididymis is a single, highly convoluted tubule connected to the testis by a series of efferent ducts. The function of the epididymis is to bring testicular spermatozoa, that are non-motile and incapable of fertilization, to functional maturity. How this maturation process is accomplished by the epididymis is not fully understood. The epididymis secretes proteins and other molecules that comprise the fluid in the epididymal lumen that bath the sperm. The components of this fluid work together to modify the sperm and bring it to maturity.

The epididymis, vas deferens and seminal vesicles have a common origin from the mesonephric (Wolffian) duct. Initially formed as the early embryonic excretory system, the mesonephric system is comprised of a longitudinal duct and a series of tubules that branch from the duct toward the developing gonad. Although most will degenerate, several of these tubules persist and anastomose with the confluence of the seminiferous tubules (rete testis), to form the efferent ducts through which spermatozoa exit the testis. The portion of the mesonephric duct closest to the efferent ducts elongates, becomes extensively convoluted, and forms the epididymis. The epididymis remains in close contact with the testis and descends with the testis into the scrotum.

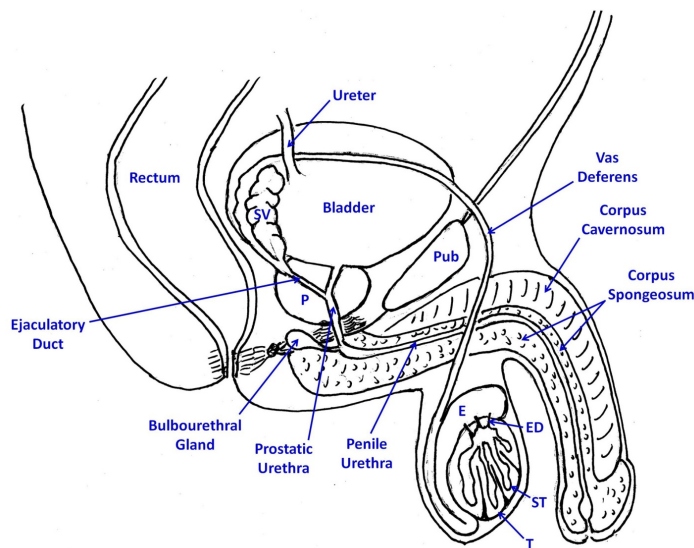


FIG. 1. Overview of the anatomy of the male reproductive track. E, epididymis; ED, efferent duct; P, prostate; SV, seminal vesicle; ST, seminiferous tubule; T, testis.

### Vas deferens

A portion of the mesonephric duct distal to the forming epididymis becomes thickened and muscular, and forms the vas deferens (or ductus deferens). In its course the vas deferens ascends from the scrotum, with the vessels that vascularize the testis and epididymis, passes through the inguinal canal, crosses behind the bladder to enter the prostate. The ejaculatory duct, also a mesonephric duct derivative, connects the vas deferens to the prostatic urethra. The primary function of the vas deferens and ejaculatory duct is transport of mature spermatozoa and seminal vesicle secretions (discussed below) to the prostatic urethra.

### Seminal vesicles

The fully developed seminal vesicle resides immediately above the prostate gland. It develops as an out-pocketing from the mesonephric duct, just proximal to the developing ejaculatory duct. The seminal vesicle is

comprised of a series of tubular alveoli, lined with a very active secretory epithelium. In fact, the seminal vesicle contributes the majority of the fluid volume of the ejaculate (~70%). Seminal vesicle secretions are rich in fructose and prostaglandins. The seminal vesicle also produces several androgen-dependent secretory proteins that are involved in such processes as the clotting of the ejaculate and immunoprotection of the sperm.

### Prostate

The prostate gland is located in the space inferior to the bladder. Its location immediately anterior to the rectum allows the prostate to be palpated and biopsied through the rectum. The prostate arises from several distinct sets of tubules that evaginate from the primitive urethra, each developing into a separate lobe. The lobes are composed of alveoli, lined with a secretory epithelium, that drain through a series of converging tubules into the prostatic urethra. The lobes are essentially continuous in the normal adult prostate, with no apparent gross or morphologic distinctions. A more recent and useful subdivision of the prostate distinguishes prostatic zones based on morphologic and functional properties (i.e., central, peripheral and transitional zones).

Prostatic secretions contribute significantly to the fluid volume of the ejaculate (~25%). These secretions are high in zinc, citric acid and choline, as well as several secretory proteins including acid phosphatase, seminin, plasminogen activator and prostate specific antigen (PSA). The exact roles of most prostatic secretions are unknown, although they are presumed important for the function of spermatozoa during and after ejaculation. Many of the proteins are proteases that are involved in the liquification of coagulated ejaculate. Elevated levels of PSA in the blood is often diagnostic of abnormal prostatic growth such as occurs with cancer of the prostate.

### Penis

The penis is responsible for the delivery of male germ cells to the female tract during sexual intercourse. It is comprised of two corpus cavernosi and the corpus spongiosum. The corpus cavernosi are erectile tissue which when filled with blood produce the penile erection. The corpus spongiosum, also an erectile tissue, forms the penile urethra and glans penis. The penile urethra is continuous with the prostatic and membranous portions urethra, and provides the remaining conduit for the sperm and ejaculatory fluids as they leave the body. The physiology of penile erection (discussed in a subsequent chapter) is complex and is subject to a number of clinical disorders. The

importance of proper erectile function to sex and reproduction, and the common occurrence of erectile dysfunction (affecting 10-20 million men in the United States), has made erectile dysfunction a primary clinical concern in andrology.

### **Endocrine and nervous control of the male reproductive tract**

The entire male reproductive tract is dependent on hormones for proper function. The pituitary produces the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), under the control of the hypothalamus. FSH is required for the initiation of spermatogenesis, and LH stimulates androgen production by the testicular Leydig cells. The testis requires high concentrations of testosterone to maintain the process of spermatogenesis and the accessory organs are dependent on androgen for proper secretory function. In addition to hormonal control, the reproductive organs are also subject to sympathetic and parasympathetic nervous control. This is particularly true for the erectile function of the penis, that is under parasympathetic control, and for ejaculation, that is under sympathetic control.

### **Conclusion**

This brief introduction to the male reproductive tract demonstrates the integrated nature of the system. The seminiferous tubules are continuous with the penile urethra via the epididymis and vas deferens, with the accessory organs contributing their secretions along this course. The entire system is maintained by pituitary gonadotropins and androgens secreted by the testis. Understanding the anatomy and embryological development of the components of the male reproductive tract are key to understanding its normal function, as well as the common, and the not-so-common, disorders encountered in the clinic.

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## **What is the relationship among the various endocrine components of the male reproductive system?**

### *Hypothalamic-Pituitary-Testicular Axis-feedback loops*

R.S. Swerdloff

The hypothalamic-pituitary-testicular unit is an integrated system that assures the adequate and appropriate secretion of male hormones and the production of sufficient sperm for the propagation of the species. Each anatomical site is integrated with the others in a classic endocrine-feedback manner, with ample local paracrine and intracrine modulation required for its most effective function.

### **Testes**

The Leydig cells of the testes are the site of production and secretion of the hormone testosterone. Through its direct action and that of its metabolites, dihydrotestosterone and estradiol, the hormonal milieu required for male sexual development and function is created; there is also a wide range of androgen-and estrogen-mediated effects on critical target organs such as the brain, bone, muscle, liver, skin, bone marrow, adipose tissue and immune systems. The Leydig cells are regulated by circulating levels of luteinizing hormone (LH), a hormone produced in the pituitary gland under the control of a hypothalamic hormone, gonadotropin releasing hormone (GnRH). GnRH in turn is modulated by neurotransmitters produced in the brain and circulating hormones. The spermatogenic compartment consists of Sertoli cells and germ cells in the seminiferous tubules, that act in an integrated fashion with one another and Leydig cells to result in normal germ cell production. The Sertoli cells are stimulated by intratesticular testosterone and follicle stimulating hormone (FSH). The testes, through their production of steroid and peptide secretory substances, also provide the regulatory feedback control of the hypothalamic and pituitary components of the axis.

### **Hypothalamic regulation of gonadotropin-releasing hormone**

#### *Hypothalamus*

The hypothalamus is the principal integrative unit responsible for the normal pulsatile secretion of GnRH, that is delivered through the hypothalamic-hypophyseal portal blood system to the pituitary gland. The

pulsatile release of GnRH provides the signals for the timing of the release of LH and FSH, which under normal circumstances occurs approximately every 60–90 minutes.

#### *Pituitary*

GnRH acts by binding to the GnRH receptors on the surface of the pituitary LH and FSH secreting cells. The secretion of GnRH is regulated in a complex fashion by neuronal input from higher cognitive and sensory centers and by the circulating levels of sex steroids and peptide hormones such as prolactin, activin, inhibin, and leptin. The local effectors of GnRH synthesis and release include a number of neuropeptides, opioids, catecholamines, indolamines, nitric oxide and excitatory amino acids,  $\gamma$ -aminobutyric acid (GABA), dopamine, neuropeptide Y, vasoactive intestinal peptide (VIP), corticotropin-releasing hormone (CRH), and kisspeptin. Catecholamines, excitatory amino acids, and nitric oxide in physiologic amounts are stimulatory, whereas kisspeptin, opioid peptides and  $\beta$ -endorphin are inhibitory. Testosterone, either directly or through its metabolic products (estradiol and dihydrotestosterone), has predominantly inhibitory effects on the secretion and release of GnRH, LH, and FSH in the male. The inhibitory effects of testosterone and estradiol on gonadotropin secretion is mediated by inhibition of kisspeptin production in the hypothalamus. Prolactin is a potent inhibitor of GnRH secretion, thus explaining its role in inhibiting LH and testosterone secretion in the clinical condition of hyperprolactinemia.

### **Integration of the hypothalamic-pituitary gonadal axis**

The normal pulsatile secretion of LH and FSH is principally driven by the pulses of GnRH from the hypothalamus. Regulation of LH and FSH is the result of feedback inhibition of the hypothalamic-pituitary component by the secretory products of the Leydig cells and Sertoli cells. Testosterone, directly and through its metabolites, regulates LH and FSH. Thus, if serum testosterone is elevated, LH and FSH will be inhibited; if testosterone is low due to Leydig cell dysfunction, LH and FSH will be increased. This is referred to as primary hypogonadism. FSH is also regulated by other Sertoli cell products; inhibin is a suppressor of FSH. If Sertoli cells are dysfunctional, spermatogenesis may be hindered and an elevated FSH may be a marker for such injury. Some patients with infertility will have reduced inhibin and isolated elevations of serum FSH. If the defect in steroidogenesis or spermatogenesis is the result of decreased LH and FSH secretion, then low serum levels of testosterone will not be accompanied with elevated gonadotropins.

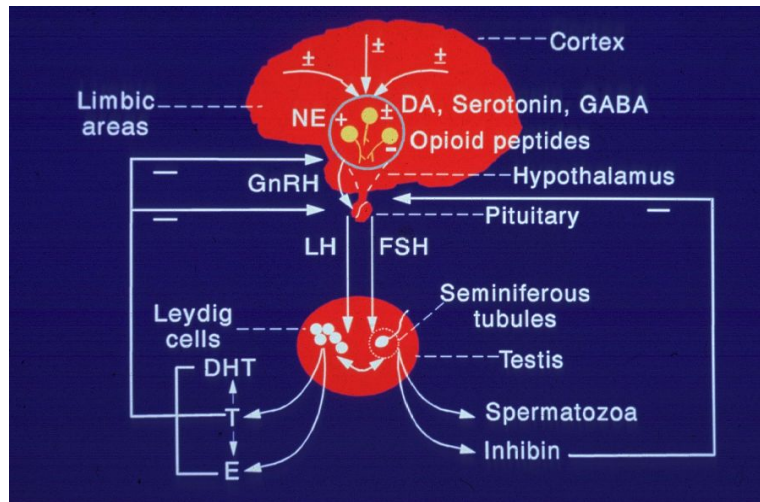


FIG. 1. Schematic representation of the components of the hypothalamic-pituitary-testicular axis and of its feedback regulators.

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## How are communication signals read in the male reproductive system?

### *Receptors for gonadotropins and androgens*

I. Huhtaniemi

The main regulatory signals of the male reproductive system are the two pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that are essential for the maintenance of testicular sex hormone production and gametogenesis. FSH stimulates the prepubertal proliferation of Sertoli cells, and in adults it controls a variety of their metabolic functions that indirectly maintain spermatogenesis. LH exerts its action on Leydig cells by stimulating their production of testosterone. Testosterone or its more potent androgenic metabolite, 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT), stimulates spermatogenesis in concert with FSH through effects on Sertoli cell metabolism. Testosterone and 5 $\alpha$ -DHT also have extragonadal actions on the differentiation, growth and mature functions of accessory sex organs (e.g. prostate and seminal vesicle). Besides gonadotropins and sex steroids, an array of other hormones and growth factors, found either in the circulation (e.g. prolactin, glucocorticoids, thyroid hormone; endocrine action) or coming from neighbouring cells (e.g. various growth factors, prostaglandins; paracrine and autocrine action) exert regulatory actions on the reproductive system. However, there is apparently much redundancy in the para/autocrine regulation, and the physiological importance of any one single factor is difficult to demonstrate.

We describe here the cellular mechanisms of action of the two main hormonal regulators of the male reproductive system, i.e. the gonadotropins and the androgen testosterone.

### **Gonadotropin Receptors**

FSH and LH are dimeric glycoprotein hormones secreted by the anterior pituitary gland. They bind to their cognate receptors that are located on cell membrane of Sertoli and Leydig cells, respectively. Because of protein structure of the gonadotropins they are not able to enter cells; therefore their contact with target cells has to occur through receptors residing on cell membranes. The contact triggers inside the target cell the formation of a "second messenger", that, in the case of gonadotropin action, is cyclic adenosine monophosphate (cAMP). The key events in the signalling mecha-

nism are demonstrated in Figure 1. Besides the classical cAMP-mediated signalling, gonadotropins also activate other signalling mechanisms, such as calcium flux, protein kinase C, MAP kinase and PI3 kinase, but their importance in the overall gonadotropin action have not yet been elucidated.

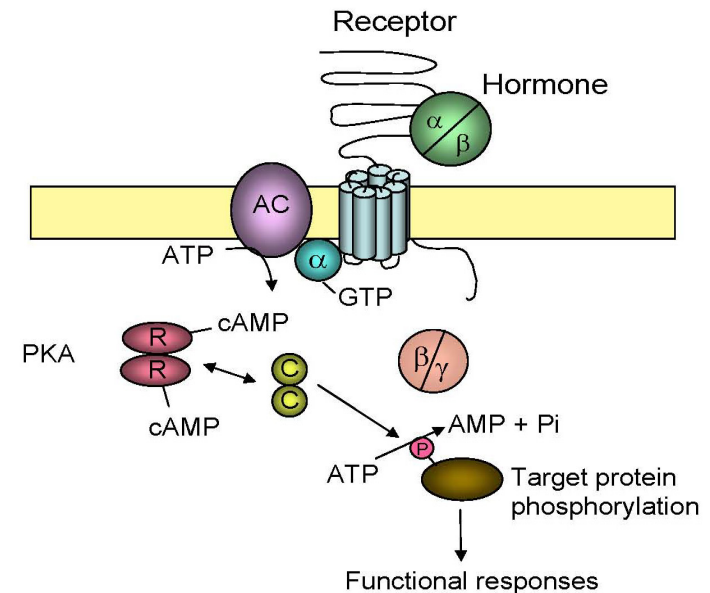
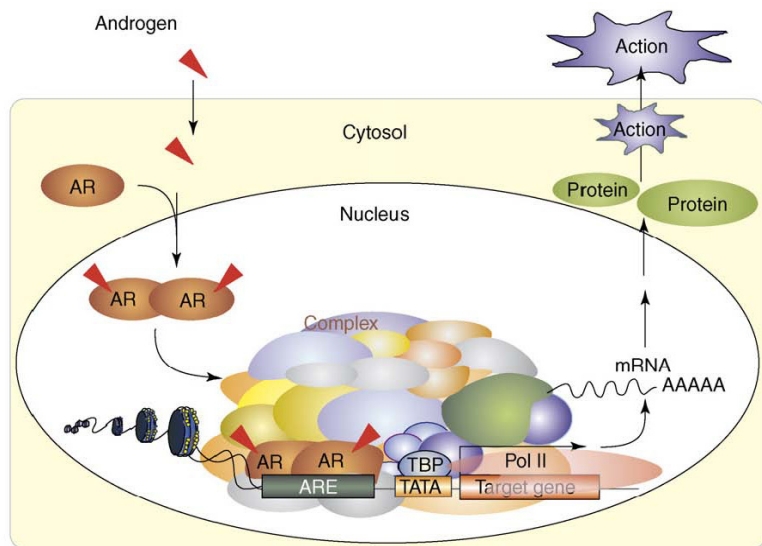


FIG. 1. **The mechanism of gonadotropin action.** LH or FSH (Hormone) bind to the extracellular domain of their cognate receptor, i.e. LH- or FSH receptor. Both are 7-transmembrane domain G-protein associated receptors (GPCRs) with long extracellular ligand-binding domain. Ligand binding induces association of guanidine triphosphate (GTP) with the  $\alpha$ -subunit of the heterotrimeric (alpha/beta/gamma) G-protein, thus activating the cell membrane associated adenylyl cyclase (AC) enzyme. The latter catalyzes the conversion of ATP to cyclic (c) AMP, which is the intracellular second messenger of gonadotropin action. cAMP binds to the regulatory subunit (R) of the tetrameric protein kinase A (PKA) enzyme. The liberated catalytic subunits (C) of PKA thereafter catalyze phosphorylation of target proteins (structural protein, enzymes, transcription factors), leading to alterations in their level of activation; this constitute the functional responses of target cells to gonadotropin action.

Androgens utilize a different principle of hormone action. Being small lipid-soluble molecules, steroid hormones can enter through the plasma



membrane into their target cells. For this reason, their receptors are located inside the cell, either in the cytoplasm or in the nucleus. Androgen receptor belongs, together with other steroid receptors, to the superfamily of ligand-activated transcription factors. Upon binding of testosterone or 5 $\alpha$ -DHT, which may occur either in the cytoplasm or nucleus, the androgen receptor binds as homodimer to specific DNA elements of promoter regions of the androgen target genes, thus acting as transcription factors. The main events in this activation process are described in Figure 2.



**FIG. 2. Mechanism of androgen action.** Androgens enter their target cell and bind to the cognate androgen receptor (AR), a ligand-activated transcription factor. After ligand binding AR will be homodimerized and localized from cytosol to nucleus, where it recognizes and binds to a specific DNA motif, the androgen response element (ARE) in the promoter region of androgen target genes. In addition, the binding of a number of co-regulators, forming the co-regulator complex, is required for androgen-bound AR to support ligand-dependent transcriptional control, which also involves chromatin remodeling and histone modifications. The consequence is increased (sometimes decreased) transcription and translation of the androgen response genes, with subsequent functional alterations of the target cell. Abbreviations: TBP, TATA-box-binding protein; TATA, TATA box; Pol II, RNA polymerase II (from Kimura et al. 2007).

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## **What compounds mediate communication within the testis? Where and how are male-associated hormones produced?**

### *Integration of the hypothalamus, pituitary and testis*

B.R. Zirkin

The two major functions of the testis are the generation of gametes and the synthesis and secretion of testosterone. Luteinizing hormone (LH), released from the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus, binds to G protein-coupled receptors on the cytoplasmic membrane of Leydig cells in the interstitial compartment of the testis, and thus stimulates these cells to convert cholesterol to testosterone. We have known for decades that testosterone is essential for the initiation, maintenance and restoration of quantitative spermatogenesis. There is good evidence that the germinal cells do not respond directly to androgens. Rather, the major target cells for testosterone within the testis are the androgen receptor (AR)-containing Sertoli cells. Follicle-stimulating hormone (FSH), also produced by the anterior pituitary, also acts on Sertoli cell receptors to promote spermatogenesis. Thus, for proper gamete production, there must be integration of the hypothalamus, pituitary and testis.

### **Mechanisms of action of testosterone and FSH**

The mechanism by which testosterone acts to regulate spermatogenesis remains poorly understood. The biological functions of androgens are considered to be mediated through the AR protein. However, much more testosterone within the testis is required for quantitative spermatogenesis than is needed for saturation of AR. Thus, it is unclear how testosterone might act as a local regulator of spermatogenesis unless, perhaps, the bioavailable testosterone concentration is reduced considerably from its total concentration. This is possible because androgen binding protein, produced by Sertoli cells, competes with AR for binding of androgen. Whether or not this mechanism reduces available testosterone sufficiently to explain the requirement for the exceptionally high concentrations required for spermatogenesis is unclear. Indeed, the possibility that testosterone has non-androgen receptor-mediated actions is possible.

FSH, too, stimulates a variety of functions in Sertoli cells, including the synthesis of secreted proteins (e.g. transferrin). In response to FSH, Sertoli

cells also produce inhibin which, along with testosterone, is involved in feedback regulation of pituitary function. FSH plays a significant role in the initiation of spermatogenesis at puberty. Its role in the adult spermatogenesis of some species (e.g. rat) is uncertain, but it plays a highly significant role in spermatogenesis in man and non-human primates.

### **Paracrine regulation of spermatogenesis**

Germ cell development involves maintaining a pool of stem spermatogonia through renewal divisions, the differentiation and mitotic divisions of spermatogonia, meiosis and spermiogenesis. As the germ cells divide and differentiate, they interact with the Sertoli cells and influence Sertoli cell gene expression. Some of the genes encode growth factors that are able to regulate germ cell differentiation, replication and survival. Thus, the Sertoli cells mediate the effects of testosterone and FSH, respond to paracrine and juxtacrine signals from the germ cells, and produce growth factors that are themselves involved in the maintenance and control of germ cell growth and differentiation.

The self-renewal and differentiation of the undifferentiated stem spermatogonial cells must be regulated so that the cells are not depleted. The Sertoli cell products kit ligand/stem cell factor (KL) and glial cell line-derived neurotrophic factor (GDNF), working together, are among the growth factors that play key roles in regulating the survival, renewal and differentiation of spermatogenic cells. Stem spermatogonia, the targets for GDNF, express the GDNF receptors Ret and GFR $\alpha$ 1. In transgenic mice, loss of spermatogonial stem cells as a consequence of their differentiation occurs when GDNF is under-expressed, and repression of the differentiation of stem spermatogonia occurs in mice in which GDNF is over-expressed. Unlike GDNF, KL has many different target cells in the mature testis; its receptor, Kit, is expressed at high levels in differentiating spermatogonia, but also in meiotic cells. KL has been shown to act as a survival factor for spermatocytes and spermatids. KL and GDNF also may be mediators of the effects of testosterone and/or FSH on spermatogenesis. Further complicating matters is that the expressions of GDNF and KL themselves appear to be regulated both by paracrine signals from germ cells and by hormones.

A number of other growth factors are expressed in the testis, but their role(s) in spermatogenesis, if any, is (are) uncertain. Insulin-like growth factor-I (IGF-1) receptors are localized to germ cells, suggesting that IGF-I may act on these cells. Transforming growth factor- $\alpha$  (TGF- $\alpha$ ), TGF- $\beta$ , and interleukin-1 (IL-1) are expressed, as are bone morphogenic protein 8a (BMP8a) and BMP8b. A knockout of BMP8b was shown to affect both the initiation and maintenance of spermatogenesis, but to not result in infertility. Basic fibroblast growth factor (bFGF), produced by pachytene

spermatocytes, is required for the *in vitro* replication of stem spermatogonia in some, but not all, strains of mice. However, a gene knockout resulting in loss of all three basic FGF isoforms did not cause infertility.

Peritubular myoid cells, Sertoli cells, Leydig cells, and spermatogonia produce leukemia inhibitory factor (LIF). Receptors for this cytokine are present on spermatogonia. *In vitro*, LIF stimulates DNA synthesis and promotes the survival of spermatogonia. However, a germ cell-specific knockout of GP130, the shared receptor for the LIF/IL-6 family of cytokines, did not cause male infertility. *In vitro*, the Sertoli cell products activin A and inhibin A stimulate and repress DNA synthesis, respectively, by spermatocytes. This may explain why male mice lacking the activin type IIA receptor, though fertile, have less efficient spermatogenesis. Finally, Sertoli cells secrete insulin-like growth factor-1 (IGF1) in response to FSH. However, no deficits in spermatogenesis were detected after IGF1 knockout. Thus, while many growth factors are expressed in the testis, to date only the Sertoli cell products KL and GDNF have been identified as essential for male fertility. These two growth factors, acting in parallel on different target cells, may define the size of a stem cell pool and thus play integral roles in spermatogenesis. However, these observations notwithstanding, it is not clear whether germ cell development represents a cascade of factor-mediated events between Sertoli and germ cells, a genetically predetermined process with only limited modulatory function for the various factors, or a combination of the two. Thus, the presence of particular growth factors might provide an environment needed for the survival and normal development of genetically pre-determined germ cells. Alternatively, there might be a series of signals delivered to Sertoli and germ cells that mediate specific events during spermatogenesis.

Cell-cell interactions in the testis also are required for the maintenance and control of cellular growth and differentiation. In the absence of macrophages, that are in close physical association with Leydig cells in the interstitial compartment of the testis, Leydig cells fail to develop or function normally. However, when macrophages are activated to produce inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), Leydig cell steroidogenesis can be inhibited. Macrophages also produce reactive oxygen species that also can have adverse effects on Leydig cell steroidogenesis. Within the seminiferous tubular compartment, incomplete cytoplasmic divisions lead to cytoplasmic bridges between and among developing germ cells, allowing clones of cells to coordinate mitosis, meiosis and differentiation. Tight junctions between Sertoli cells prevent the passage of macromolecules from the interstitium into the tubule lumen. Sertoli cells provide lactate and pyruvate to support germ cell metabolism, as well as transferrin and ceruloplasmin transport iron and copper to germ cells. In turn, developing germ cells influence Sertoli cell function, including

stimulation of ABP production that, in turn, may affect testosterone action. There also may be peritubular cell-Sertoli cell and Leydig cell-Sertoli cell interactions. For example, damage to the seminiferous tubules by cytotoxic agents, vitamin A deficiency, irradiation or cryptorchidism alters Leydig cell morphology, and Sertoli cell-conditioned media has been shown to influence Leydig cell testosterone production. Inhibin and activin, produced by Sertoli cells, both can influence Leydig cell testosterone production *in vitro*, as can growth factors produced by Sertoli cells.

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## How is the synthesis of testosterone regulated?

V. Papadopoulos

The ability of a cell to make testosterone is defined by the presence of the steroidogenic machinery in an appropriate cellular environment able to respond in an optimal manner to the received stimuli. Testosterone, the major male sexual hormone, is synthesized and secreted primarily by Leydig cells of the testis. In these cells, testosterone synthesis is under the tight control of the pituitary gonadotrophin luteinizing hormone (LH), and also is influenced by local factors coming from other testicular cells or from external factors such as drugs and environmental chemicals.

There are a number of key points that are critical for the establishment and optimal function of the steroidogenic machinery responsible for testosterone synthesis in the Leydig cell (Figure 1). These include: (i) integrity of the plasma membrane LH receptor signal transduction cascade responsible for sensing and responding to the blood-borne LH; (ii) availability of sufficient amounts of the substrate cholesterol coming from the blood or synthesized *de novo*; storage or immediate availability of cholesterol for use in androgen production; (iii) de-esterification of cholesterol thus making available the stored cholesterol for androgen formation; (iv) integrity of the mechanism responsible for transporting cholesterol from intracellular stores into mitochondria; (v) availability of appropriate levels and combinations of the nuclear transcription factors controlling the expression of proteins involved in cholesterol transport and in testosterone biosynthesis; (vi) maintenance of appropriate organelle structures required for optimal testosterone formation; (vii) appropriate spatial and temporal expression of steroidogenic enzymes, mainly cytochrome P450 monooxygenases and dehydrogenases, responsible for metabolizing cholesterol to various intermediates leading to testosterone formation; and (viii) availability of the co-factors necessary for steroidogenic enzyme action. Steroids leave Leydig cells by passive transport. Steroids in the circulation exist as free or bound to extracellular proteins such as sex hormone binding globulin (SHBG), androgen binding protein (ABP) and/or albumin.

For Leydig cells to respond to LH and to function optimally, it is critical that the proteins involved in steroidogenesis, from the LH receptor to cholesterol transporting proteins and steroidogenic enzymes, are synthesized without any mistake in their amino acid sequences. Mutations of the involved proteins can be lethal or lead to disease states such as pseudohermaphroditism, hypogonadism, and infertility.

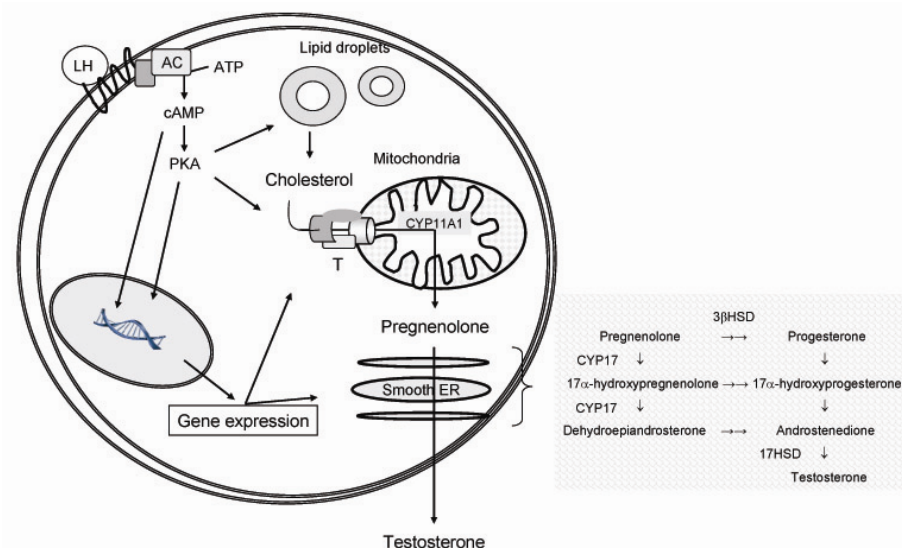


FIG. 1. Schematic representation of the steps involved in testosterone formation. Blood-borne luteinizing hormone (LH) binds to a G-protein coupled receptor leading to activation of adenylate cyclase (AC) that converts ATP to cAMP, the major second messenger of LH action in Leydig cells. cAMP subsequently activates the cAMP-dependent protein kinase (PKA), an event that triggers a series of reactions including the de-esterification of cholesterol from lipid droplets and activation of proteins, PKA substrates, involved in cholesterol transport into mitochondria. cAMP, as well as PKA act also in the nucleus activating steroidogenic protein and enzyme gene expression. Free cholesterol is transporter and imported into mitochondria via a multiprotein complex (T for transduceosome) formed to amplify the effect of LH and cAMP. This complex included proteins such as the A-kinase anchoring proteins PAP7 and AKAP121, steroidogenesis acute regulatory protein (StAR), translocator protein (18-kDa), and voltage-dependent anion channel. Imported cholesterol is metabolized to pregnenolone by the cytochrome P450 side chain cleavage (CYP11A1). Pregnenolone is subsequently metabolized in the smooth endoplasmic reticulum by a series of enzymes (CYP17, 3β-hydroxysteroid dehydrogenase and 17-hydroxysteroid dehydrogenase) to form testosterone.

The amount of testosterone formed by the Leydig cells reflects specific needs of the body for cell growth, organ formation, masculinization and maintenance of androgen-dependent functions. During development, the differentiation of Leydig cell precursors leads to the establishment of specific

Leydig cell populations that are responsible for the formation of the amounts of testosterone needed at various ages. In the fetus, testosterone-forming fetal Leydig cells function in the prenatal masculinization of the male urogenital system. After birth this population disappears, and the adult Leydig cell population, evolving in an environment free of maternal factors, develops from a small pool of undifferentiated, self-renewing stem Leydig cells via a sequence of stages that include Leydig cell progenitors, immature Leydig cells and adult Leydig cells. These changes reflect the need for testosterone in the development of male characteristics, puberty and androgen-dependent functions. Changes in cell structure and gene expression are responsible for the morphological and functional differences among these various cell types; the basic components of the steroidogenic machinery are present but not used to the same extent. Thus, testosterone production changes during development are due to alterations in the cellular environment, and are designed for the optimal production of testosterone to accomplish specific needs. In aging, various components of the steroidogenic machinery fail to function at an optimal level, leading to a decline in androgen formation.

Although it seems that the pathway of testosterone formation and its regulation by LH are well established, there are many steps that have not been yet defined and questions have been raised about the adopted mechanisms leaving the field open for further investigation.

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## How are germ cells produced and what factors control their production?

M. Nagano

With every heartbeat, a man produces ~1,000 sperm. At this rate, 72,000 sperm are generated in a minute, over 100 million in a day, and 3 billion in a month. Furthermore, male gametogenesis (spermatogenesis) continues for almost the entire life. Thus unlike women, men can father genetic children naturally at more advanced age beyond 60 yrs. How does the male reproductive system achieve this remarkable functionality?

The key to understand the biological mechanism of spermatogenesis is to understand the nature and function of stem cells. Indeed, other self-renewing tissues in our body that are able to replenish and maintain the structure and function of different organs rely on proper functioning of stem cells. Examples include our hematopoietic system, skin and intestinal epithelium. We have over 10 trillion erythrocytes in our blood circulation at any given time of our life despite a limited life-span of 120 days for each cell. Similarly, cells of the skin and intestinal epithelia die and slough off constantly. Yet these epithelia remain functional for a lifetime. As such, in these systems (so-called self-renewing tissues), cells are continuously lost but replenished to maintain the structure and function of an organ throughout life. This is made possible by stem cells, and spermatogenesis is one such self-renewing system supported by stem cells.

Stem cells of spermatogenesis are called spermatogonial stem cells (SSCs). They are part of the spermatogonia (diploid male germ cells). During steady-state spermatogenesis, one SSC divides into two cells: one remains as a stem cell (self-renewal) and the other differentiates to eventually become spermatozoa (differentiation) (FIG. 1). The one that has self-renewed is re-incorporated in the stem cell pool while the one that has taken a differentiation path is considered “lost”, as it leaves the testis for the reproductive tract after becoming spermatozoa. How an SSC chooses which path to take, a phenomenon termed “fate decision”, is currently the focus of intensive research.

SSCs are a sub-population of spermatogonia; thus, they are found within the seminiferous tubules, residing on the basal membrane and surrounded by Sertoli cells. In general, a microenvironment that contains stem cells is called a stem cell “niche”; it supports and regulates stem cells. The SSC niche is composed of Sertoli cells, the basal membrane, and daughter germ cells that SSCs have produced. Does the niche affect SSC activity? Although there is no strong evidence that hormones, e.g., FSH, LH, testosterone,

directly control SSCs, it is known that Sertoli cells secrete a soluble protein factor, GDNF (glial-cell-line-derived neurotrophic factor), that is important for SSCs to self-renew. If a functioning GDNF gene is found only in one allele in a mouse, SSC numbers become significantly reduced. In the preparation of an SSC culture, GDNF is an indispensable factor to maintain self-renewal of SSCs. Additional stimulators of SSC self-renewal include FGF2 from Sertoli cells and CSF1 (colony-stimulating factor 1) from Leydig cells and select peri-tubular myoid cells. Most investigators believe that there exist additional factors that can control and regulate SSC activity. More players of SSC fate regulation are expected to be identified in the future.

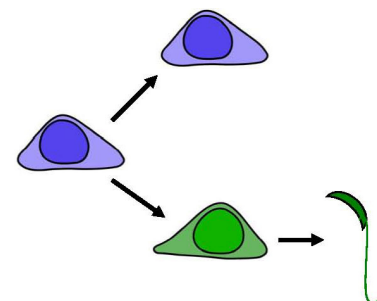
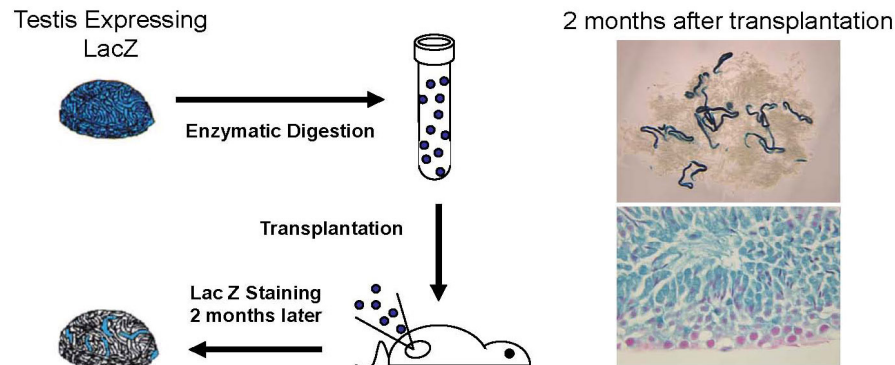


FIG. 1. **Self-renewal and differentiation of SSCs.** SSCs self-renew to maintain a stem cell pool. They can also commit to differentiation to produce sperm. Blue = SSCs. Green = committed male germ cells.

To maintain spermatogenesis at a steady state for daily sperm production, the input (stem cell differentiation) and output (the loss of spermatozoa from the testis) need to be balanced. It is known, however, that SSCs are very few (0.01 – 0.02 % in an adult mouse testis) and divide very infrequently. To compensate for these short-comings of SSCs, it is assumed that SSCs produce a subpopulation of spermatogonia termed “amplifying compartment” that divide more frequently to expand the size of the population.

SSCs are defined functionally by their ability to continuously self-renew and differentiate. In other words, they cannot be identified convincingly by morphology or biochemical / molecular properties at present. As SSCs cannot be visualized microscopically, the detection of SSCs requires a bio-assay that measures the stem cell function of self-renewal and differentiation. Spermatogonial transplantation is an example of such a bio-assay and has been developed using the mouse (FIG. 2). In this technique, cells derived from a donor testis are injected into the seminiferous tubules of sterile

recipients (no germ cells in their testes). Two months later, donor SSCs regenerate spermatogenesis in segments along the recipient seminiferous tubules. The regeneration of donor-derived spermatogenesis implies that there were cells in the donor testis cell preparation that were able to self-renew and differentiate for a long time. Since these are the activities unique to stem cells, successful regeneration and maintenance of spermatogenesis indicate the presence of SSCs in donor cells. This assay is analogous to the detection of gravity; gravity is invisible, but we can detect its presence through experimental intervention by dropping an object in the air. A problem of this SSC assay is that it detects SSCs only retrospectively and does not allow us to determine which cells are SSCs before transplantation. Therefore, researchers are now actively searching for marker molecules expressed specifically by SSCs to identify these cells prospectively; indeed, some markers have been discovered.



**FIG. 2. Spermatogonial transplantation.** The left side of the figure shows the transplantation procedure. The donor testis is derived from a transgenic mouse carrying the bacterial LacZ gene. The donor testis is digested into single cells using enzymes. These cells are injected into the seminiferous tubules of a recipient mouse that is infertile. Two months later, recipient testes are harvested and reacted with LacZ substrate, which makes donor-derived cells blue. As shown in the right panels, SSCs derived from the donor testis regenerate spermatogenesis in the form of blue segments along the recipient seminiferous tubules, which is visualized by the LacZ reaction (blue). Each segment is made of fully regenerated spermatogenesis that the donor SSCs produced.

SSCs are expected to be a powerful resource to restore male fertility in the future. For instance, cancer treatment with chemotherapy is toxic to male

germ cells including SSCs. Thus, even if the cancer is cured, the patient may lose his fertility. This treatment complication is of great concern for prepubertal boys who have not started spermatogenesis and therefore cannot benefit from sperm banking. Since SSCs exist from the time of birth, SSCs can be harvested from the testes of a patient at any age to be cryopreserved before chemotoxic therapy. When the patient is ready to have his own child, cryopreserved SSCs may be thawed and transplanted back into his testes. The transplanted SSCs, which were harvested before exposure to chemotherapeutic drugs, will regenerate spermatogenesis and produce sperm, making it possible to achieve conceptions through natural intercourse or with the use of assisted reproductive technologies. Further studies on SSC biology may allow this fertility preservation strategy to become a reality in the near future.

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## What unique chromosomal events lead to the formation of a haploid male germ cell?

M.A. Handel

Meiosis is a unique and defining event of gametogenesis serving at least two functions in the reproductive life cycle: it reduces chromosome number to the haploid state in the gamete (thus allowing diploidy to be restored at fertilization), and it shuffles gene allele combinations, giving rise to genetic diversity. Meiosis is accomplished in two remarkably coordinated divisions, without an intervening S phase (Fig. 1). The first division, a reductional division, segregates the homologous chromosomes into separate cells, and the second division, an equational division, reduces the DNA content to haploidy. The success of these divisions depends on the unique dynamics of chromosomes during the extended meiotic prophase.

Meiosis is initiated after mitotic proliferation of spermatogonia by DNA synthesis that accomplishes precise replication of each chromosome to form two chromatids. Thus the DNA content ("C" value) has doubled from 2C to 4C, but the chromosome number ("N" value) of the germ cell is not changed – it is still the 2N diploid value, e.g., 46 chromosomes in humans (note: spermatocytes are not tetraploid). During meiosis I prophase, homologous chromosomes pair, forming bivalents, undergo recombination – these are defining events of meiosis and key features that distinguish meiosis from mitosis. Meiotic prophase I is divided into substages that mark the dynamics of chromosome behavior. During the leptotene stage, chromosomes are subject to endogenous double-strand DNA breaks, mediated by the SPO11 enzyme, that initiate the molecular events of meiotic recombination. Also during the leptotene phase, homologous chromosomes find each other by homology searching mechanisms that are not well understood, but may be facilitated by telomere clustering into a "bouquet" on the nuclear envelope and/or the DNA breaks and subsequent formation of short single-stranded ends. During the zygotene stage, chromosome pairing extends and homologs become intimately associated by synapsis, a process mediated by the synaptonemal complex (SC).

The SC is a protein complex comprised of lateral elements that form the scaffold, or axes, of each homolog, and a central element that is structural "glue" mediating complete synapsis. The completion of synapsis marks the beginning of the pachytene stage, which is lengthy (approximately 16 days in the human) and characterized by considerable growth of the spermatocyte as well as by important chromosome dynamics. Now the chromosomes are visualized as homologous pairs, called bivalents, e.g., 23 bivalents in

humans. Notably, however, the non-homologous X and Y chromosomes are synapsed only in a small region of homology (the pseudo-autosomal region) and are sequestered in a heterochromatic nuclear domain known as the XY body (or sex body). During the pachytene stage, molecular events of homologous recombination unfold. Interestingly, the number of recombination-initiating double-strand breaks is in approximately ten-fold excess to the number of final reciprocal recombinations (crossovers), which is always at least one per chromosome, but can be up to two or three in longer chromosomes. The excess DNA double-strand breaks are thought to be repaired by a recombination mechanism that involves non-crossover gene conversion, or site-specific exchange of information without exchange of surrounding chromosomal regions.

The completion of recombination marks the passage of the germ cell into the final, diplotene, stage of meiosis I prophase, when the chromosomes undergo desynapsis and condense. At this stage, the homologs are still held together by the recombination sites (crossovers), visibly manifest as chiasmata. The chiasmata serve the essential function of maintaining the homologous pairs in a bi-polar orientation as they line up on the spindle apparatus at metaphase of the first meiotic division. The first meiotic division is reductional, separating the members of each homologous pair. The result is two cells, secondary spermatocytes, each with the haploid chromosome content (e.g., 23 chromosomes in humans), but with each chromosome still comprised of two chromatids. The meiosis II division ensues rapidly and is an equational division much like mitosis, separating the chromatids to separate cells, each of which now contains the haploid 1N chromosome number (e.g., 23 chromosomes in humans) and 1C DNA content. Similar to the mitotic divisions of differentiated spermatogonia, the two meiotic divisions are characterized by incomplete cytokinesis and the persistence of intercellular bridges. Thus, when meiosis is completed, the haploid round spermatids are conjoined in a syncytium as they commence the differentiation process of spermiogenesis.

Much attention has been directed to how the meiotic choreography of chromosome movement and the processes of recombination can go wrong, and whether these errors can explain any cases of human infertility. The penalty of errors is germ-cell arrest or generation of aneuploid gametes and offspring, bearing the wrong number of chromosomes. Both failure of recombination and chiasmata formation or abnormal persistence of chiasmata can negatively impact fidelity of chromosome segregation during the first meiotic division by causing nondisjunction (failure of homologs to separate to the two spindle poles). Likewise, absence or persistence of sister chromatid cohesion can cause abnormalities in chromosome segregation. Screening infertile men for gametic aneuploidy by monitoring sperm

chromosome content using fluorescent in situ hybridization (FISH) has failed to find strongly significant correlations between infertility and production of aneuploid sperm, and, indeed, meiotic nondisjunction is less frequent in the human male than in the female. However, infertility due to arrested spermatogenesis and germ cell death is a common phenotype in mouse models lacking proteins involved in meiotic recombination. Although similar mutations have been found among infertile males, causality is difficult to establish. Nonetheless, it can be anticipated that the advent of personal genome sequencing will facilitate the identification of meiosis mutations in humans.

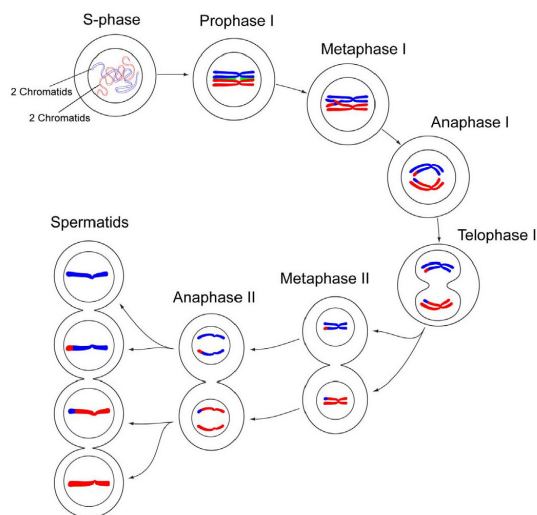


FIG. 1. This diagram illustrates the sequence of key events of meiosis in male germ cells. Red and blue depict the two homologs of an autosomal chromosome, one maternally derived and the other paternally derived. At the completion of S-phase, each chromosome consists of two chromatids. At the pachytene stage of prophase I, homologs are synapsed to form a bivalent, an event mediated by the synaptonemal complex (green). By metaphase I, chiasmata, the visible manifestation of recombination events, are seen. In anaphase I and telophase I, the homologs separate from each other, reducing the chromosome number to the haploid content. Spermatocytes rapidly progress to metaphase II, and subsequently the chromatids are separated in anaphase II, to form the 4 haploid spermatids.

In summary, meiosis is a defining event of spermatogenesis and is comprised of two divisions (Fig. 1). The events of meiosis I prophase include

pairing and synapsis of homologous chromosomes and reciprocal recombination, resulting in new combinations of gene alleles. The first of the two meiotic divisions is a reductional division, separating homologous chromosomes and reducing chromosome number from  $2N$  to  $1N$ . The second division is equational, separating chromatids. The products of these meiotic divisions are 4 spermatids, each with the haploid  $1N$  chromosome number and  $1C$  DNA content.

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## How is the production of spermatozoa regulated?

### *Hormonal regulation of spermatogenesis: role of androgens and FSH*

R.I. McLachlan

Spermatogenesis features mitotic and meiotic divisions, elaborate cytodifferentiation and changing inter-cellular interactions, that are driven by the interplay of autocrine, paracrine and endocrine factors. Endocrine systems regulate both the initiation (neonatal development and puberty) and the maintenance of spermatogenesis via a classic negative feedback system (Chapter 2, Fig. 1) involving elements of the hypothalamic-pituitary-testis (HPT) axis. Spermatozoa production is dependent on stimulation by the pituitary gonadotropins, LH and FSH, which are secreted in response to hypothalamic gonadotropin-releasing hormone (GnRH). Their action on germ cell development is effected by FSH and androgen receptors on the somatic Sertoli and Leydig cells, respectively. LH stimulates Leydig cell testosterone secretion which acts to stimulate sperm production, provide virilisation of non-gonadal tissues, and also feeds back on the hypothalamus and pituitary to regulate GnRH secretion. FSH stimulates Sertoli cells to promote spermatogenesis as reflected by their secretion of inhibin B that has a negative feedback effect on pituitary FSH secretion. The physiology of these key endocrine regulators of spermatogenesis are outlined and illustrated through consideration of hormonal contraception and treatment of infertility.

### Hormonal regulators of spermatogenesis

#### *Androgen receptor (AR)*

Androgens regulate spermatogenesis by binding to ARs expressed in Sertoli cells. Testosterone, or its 5 $\alpha$ -reduced metabolite dihydrotestosterone (DHT), bind to cytosolic AR which then dimerise, translocate to the nucleus and bind to androgen response elements in the promoters of androgen-responsive genes to modulate gene transcription, the so-called “genomic” pathway of action, although rapid “non-genomic” pathways also operate. A functional Sertoli cell AR is essential for spermatogenesis. Sertoli cells AR expression is highest in those sections for the seminiferous tubule involved in supporting meiosis and spermatid development. Numerous factors regulate AR expression including FSH, intra-testicular testosterone levels and cytokines (e.g. nuclear factor- $\kappa$ B).

#### *FSH Receptor (FSH-R)*

FSH binding of FSH-R on Sertoli cells initiates signal transduction events including cAMP stimulation and Ca<sup>2+</sup> release leading to intracellular signalling cascades, such as protein kinase-A and C dependent pathways. Sperm production can occur without FSH action in mice, rats and perhaps man, but the production of normal sperm numbers requires FSH action. The FSH-R is expressed in those sections of the seminiferous tubule involved in supporting spermatogonial mitosis.

#### *Other steroid hormones*

Estrogen receptor (ER): the testis is a major site of estrogen production and intratesticular levels are exceedingly high. Aside from ER $\alpha$  in the efferent ducts, ER $\beta$  (including splice variants) is found in human Sertoli cells and most germ cell types, yet evidence for a direct role in spermatogenesis remains elusive. Thyroid hormone receptor expression is important in Sertoli cell development.

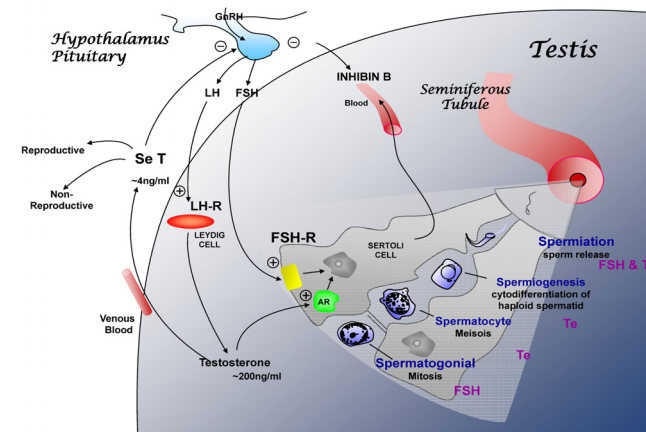


FIG. 1. **The hormonal regulation of spermatogenesis.** FSH; follicle stimulating hormone, LH; luteinizing hormone, GnRH; gonadotropin-releasing hormone, LH-R; LH receptor, FSH-R; FSH receptor, AR; androgen receptor. Note the location of FSH-R and AR on Sertoli cell that transduce these effects to complete spermatogenesis. The 4 phases of spermatogenesis are shown (mitosis, meiosis, spermiogenesis, and release of sperm into the lumen [spermiation]). The principal hormone (FSH or T) regulating each phase is indicated (although evidence for combined action exists in many models). Note also that testicular testosterone levels are exceedingly high relative those in serum, and that serum T effects androgenic actions on other reproductive and non-reproductive tissues, and provides negative feedback on pituitary gonadotropin secretion. Inhibin B exerts inhibitory effects only on FSH release.

## Hormonal dependency of human spermatogenesis

FSH and testosterone support the initiation of spermatogenesis, and are both needed for quantitatively normal spermatogenesis in adulthood. This is the physiological principle behind (i) male hormonal contraception wherein exogenous testosterone administration reduces pituitary LH and FSH secretion rendering the majority of men (reversibly) azoospermic, and (ii) combined gonadotropin therapy to restore fertility in hypogonadotropic hypogonadism (HH) that may result from a deficiency of hypothalamic GnRH drive or pituitary gonadotropin secretion. In congenital HH, germ cells do not proceed beyond the spermatogonial stage; HH acquired after puberty leads to spermatogenic regression and marked oligo/azoospermia. When HH is partial, reduced sperm output may permit fertility but when complete, spermatogenesis fails. Restoration of endogenous gonadotropin secretion or exogenous gonadotropin replacement therapy will induce/restore spermatogenesis in most cases, but may take many months.

## Gonadotropin and androgen effects on spermatogenesis

### *Testicular sex hormone physiology*

Spermatogenesis has an absolute need for androgen action. Due to its local production, intra-testicular testosterone levels in man are ~ 50 fold higher than those in serum and fall by over 98% when LH levels are suppressed. Data from rats shows that spermatogenesis can be fully maintained despite a reduction of testosterone concentrations to 20% of normal. The level of testosterone required to *initiate* spermatogenesis in the mouse is about 16 fold greater than that needed for the *maintenance*, indicating different mechanisms operate depending on maturational state.

The dissociation constant of the testicular AR is  $3 \times 10^{-9}$ M suggesting that the AR is fully saturated in the normal testis. Binding of testosterone to high affinity sex hormone-binding proteins may sequester part of the intratesticular free testosterone pool. The 5 $\alpha$ -reductase enzyme catalyses the conversion of testosterone to DHT. Despite DHT's higher affinity for the AR and higher AR-dependent transcriptional activity compared to testosterone, the vast excess of testosterone over DHT (about 100:1) in the normal testis suggests that DHT is not important. When intra-testicular testosterone are markedly reduced by LH suppression, residual intratesticular DHT may play a supportive role. It has been suggested that inhibition of 5 $\alpha$ -reductase (especially the type 1 isoenzyme present in testis) may enhance male hormonal contraceptive efficacy by augmenting androgen deprivation but evidence for this has not been forthcoming.

### *The role of FSH in human spermatogenesis*

The frequent requirement for FSH to establish spermatogenesis in congenital HH (see below) points to the need for FSH to induce permanent maturational effects on the Sertoli cell/seminiferous epithelium, as during normal puberty.

Case reports of rare men with FSH and FSH-R mutations reveal some inconsistencies in the spermatogenic phenotypes: men with FSH-R mutations have moderately elevated FSH levels, variably severe reductions in sperm counts yet those with an inactivating FSH $\beta$  mutation are azoospermic. Thus unlike the FSH $\beta$  knockout mouse, FSH may be essential in establishing / maintaining some level of spermatogenesis in man.

The dual hormonal dependency of human spermatogenesis was clearly demonstrated in the setting of experimental gonadotropin suppression: sperm production was restored to 50% of baseline values by either FSH or human chorionic gonadotropin (as an LH substitute) treatment whereas only combined hCG plus FSH treatment led to quantitative restoration. When gonadotropin levels are suppressed in normal men, as in the context of male hormonal contraceptive treatment, dramatic effects on germ cell development are evident with accelerated germ cell apoptosis and the inhibition of spermatogonial maturation and late spermiogenic progression, and the retention / degradation of spermatids within Sertoli cells.

## Conclusion

During development androgens, FSH and thyroid hormone assist to set up the essential 'infrastructure' for later spermatogenesis. In adults spermatogonial proliferation, germ cell survival, spermiogenesis and spermiation depend upon FSH and androgen signalling pathways for optimal sperm output. This knowledge has contributed to the development of better treatment of infertility due to gonadotropin deficiency and to the development of hormonal contraceptives.

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## Are there epigenetic events associated with male germ cell formation? What is the role of genomic imprinting in male germ cells?

J.M. Trasler

Epigenetics refers to heritable mechanisms of modulating gene expression that do not involve alterations in DNA sequence. Thus epigenetics affects the phenotype of a cell without affecting its genotype and is governed by processes that permit heritability from the parental to the daughter cells but are at the same time potentially modifiable or even reversible. DNA methylation, histone modifications and small RNAs are the three main molecular mechanisms that have been associated with epigenetic regulation of genes. These three mechanisms interact and stabilize each other and disruptions of one or more can lead to inappropriate expression or silencing of genes, resulting in “epigenetic diseases” such as cancer and imprinting disorders. Epigenetic mechanisms are conserved across higher eukaryotes including humans suggesting that they act in combination to regulate higher order chromatin and ultimately genome integrity.

Epigenetic patterning begins in the germline and is essential for normal embryo and postnatal development. Epigenetic modifications occurring during germ cell development are postulated to play roles in gene expression, meiosis, genomic integrity and genomic imprinting. Male and female germ cell development is a particularly critical time for the acquisition of the differential ‘marking’ of imprinted genes to ensure parent-of-origin specific expression. Genomic imprinting involves about 100 genes to date and refers to variation in the expression of these genes according to their maternal or paternal origin; imprinted genes play key roles in growth, placental function and many aspects of development including neurobehavioral processes. Many imprinted genes have distinct sequences known as differentially methylated regions or DMRs where DNA methylation differs between the maternal and paternal alleles. Since DNA methylation is the most extensively characterized epigenetic mechanism, it will be used here to illustrate the importance of epigenetics to male germ cell biology.

DNA methylation is found at the 5-position of cytosine residues within CpG dinucleotides (where cytosine is 5' to guanine) at 20-30 million sites throughout the mammalian genome; about 60-80% of cytosines within CpGs are methylated. Methylation of DNA is invariably associated with transcriptional silencing. Two types of DNA methylation occur, either *de novo* methylation or the acquisition of methylation on unmethylated cytosines, or maintenance methylation, that takes place at the time of DNA replication to

ensure the propagation of genomic methylation from parent to daughter cells. The majority of *de novo* methylation is targeted to transposons and their remnants and to repeats such as pericentric satellite sequences with smaller amounts at single-copy sequences and the DMRs of imprinted genes. Methylation of DNA is catalyzed by a family of DNA (cytosine-5)-methyltransferases (DNMT enzymes or DNMTs). The main DNMTs involved in the establishment and maintenance of methylation patterns are DNMT1, DNMT3A and DNMT3B. Although it lacks enzymatic activity, DNMT3L (DNMT3-like) is related to and works with DNMT3A and DNMT3B. Demethylation occurs passively when methylation is not maintained following DNA replication or actively by an as yet poorly understood process/enzyme.

Epigenetic modifications including DNA methylation are for the most part erased in primordial germ cells and then reacquired in a sex-specific manner during germ cell development (Fig. 1). The erasure is particularly important for imprinted genes as maternal ‘marks’ on imprinted genes inherited from the mother must be erased and new paternal ‘marks’ introduced. A second period of erasure occurs in the preimplantation embryo and is thought to affect most sequences with the exception of imprinted genes and some repeat sequences. Imprinted gene methylation is maintained during preimplantation development since it is only in the germline (male or female depending on the gene) that imprinted genes acquire the allele-specific methylation that will result in monoallelic expression in the embryo and postnatal individual. As most methylation will be erased in the early embryo, it is postulated that germline DNA methylation, at sequences other than imprinted genes and repeats, plays specific roles in germ cell development, gene expression or chromatin structure during gametogenesis.

Following erasure in primordial germ cells (at approximately mid-gestation in the fetus), most male germline DNA methylation is acquired premeiotically in the prenatal gonocytes or pro-spermatogonial cells in the period between mid-gestation and birth (Fig. 1). DNA methylation acquisition in male germ cells continues after birth in the mitotic and meiotic germ cells and is complete by the pachytene phase of meiosis. Mitotic spermatogonia must also maintain the DNA methylation patterns acquired in the prenatal period. The precise signals that target DNA methylation to specific sequences in male germ cells are unclear but may include RNA-directed events. Potential candidates for RNA-based targeting are the recently described germ cell-specific small RNAs, the piRNAs. Gene-targeting experiments in mice have identified DNMT3a and DNMT3L as the predominant enzymes involved in the methylation of repetitive and imprinted sequences in the male germline. Absence of these enzymes results in the failure of spermatogenesis and infertility. For example, in male mice lacking DNMT3L, male germ cells over-express retrotransposons, and there is

asynapsis and non-homologous synapsis during meiotic prophase and subsequent apoptosis of germ cells prior to pachytene.

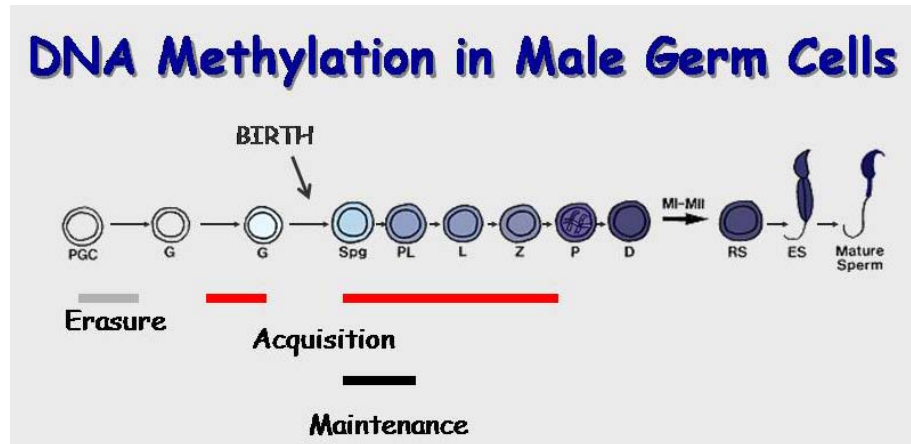


FIG. 1. Timing of the erasure (gray bar), acquisition (red bar) and maintenance (black bar) of DNA methylation patterns in the prenatal and postnatal periods of male germ cell development. PGC, primordial germ cell; G, gonocyte or prospermatogonia; Spg, spermatogonia; PL, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; P, pachytene spermatocyte; D, diplotene spermatocyte; RS, round spermatid; ES, elongating spermatid.

Evidence is accumulating that errors in the establishment or maintenance of germ cell DNA methylation patterns can cause human diseases such as imprinting disorders and cancer. Sperm samples from oligospermic patients have been reported to contain DNA methylation defects at imprinted loci. It has been suggested that paternal age effects that are seen for schizophrenia and other human conditions may involve errors in the maintenance of genomic methylation in the spermatogonia of older men. Furthermore, there is also concern for more than one generation, since while most epigenetic errors will be erased in the germline of the progeny, there are several examples of the potential for inheritance of epigenetic defects. Ongoing studies are assessing the effects of environmental conditions, diet and drugs on DNA methylation patterns in human sperm.

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## What does the epididymis do and how does it do it?

B.T. Hinton

*"If anyone asks what the epididymis is, we shall answer that it is a vessel constituting by various twists a body affixed to the back of the testicle" (de Graaf, 1668; see Jocelyn & Setchell, 1972).*

Spermatozoa leaving the testis are neither motile nor able to recognize or fertilize an egg; they must traverse a long duct, the epididymis, to acquire these abilities. These post-testicular transformations of spermatozoa are collectively called sperm maturation. The epididymis is a single highly convoluted duct/tube of approximately 1 meter in length in the mouse, 3 meters in the rat, 6 meters in the human and a remarkable 18 meters in the stallion. Hence, it can take anywhere from 1 to 14 days for spermatozoa to traverse the epididymis. Early investigators considered the epididymis as a holding tube for spermatozoa and that it was a place where spermatozoa aged. It was thought that the maturation process was inherent to spermatozoa and had little to do with the epididymis. It is now clear that the epididymis is very much an active participant in the maturation process, not only providing an appropriate luminal fluid microenvironment, but also supplying many of the molecules required by spermatozoa for the acquisition of fertility. The challenge for many investigators has been to identify those molecules. In addition to its sperm maturational role, the epididymis places a premium on protecting spermatozoa as they mature; it also provides an environment for storage following the maturation process. Since spermatozoa are immotile, they require assistance to move along this very long duct. This movement is aided by contractions of smooth muscle that surround the duct as well as pressure from fluid and spermatozoa entering the duct from the testis. From a clinical perspective, an improper functioning epididymis results in male infertility and therefore, the epididymis is considered to be a prime target for the development of a male contraceptive. Interestingly, unlike the testis and prostate, cancer is rarely observed in the epididymis.

### Structure of the epididymis

The gross anatomical structure of the epididymis in a variety of species is divided into several regions that include: the initial segment, caput and corpus and cauda regions. Proximally, the efferent ducts connect the testis to the epididymis and distally, the vas deferens extends from the cauda region

(FIG. 1). Within each region there are multiple segments separated by septa, with the numbers of segments within each region being variable. The challenge for investigators is to relate the different regions and segments to epididymal function and sperm maturation.

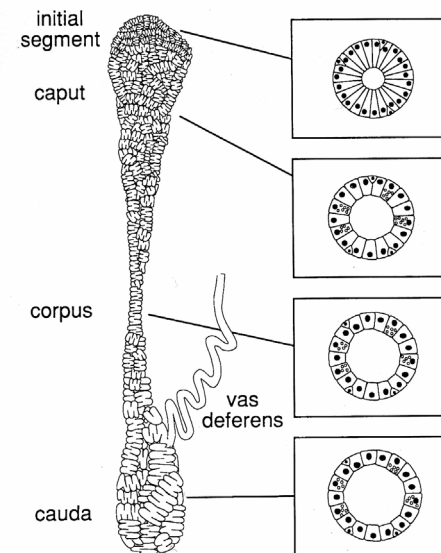


FIG. 1. Schematic representation of an epididymis showing the different regions: initial segment, caput, corpus and cauda. To the right are shown cross-sectional representations of the epididymal duct at each region. Note how the luminal diameter increases and the cell height decreases from the initial segment to the cauda.

The epithelium of the epididymis is comprised of several cell types including: principal, basal, apical, halo, clear and narrow cells, each of which vary in number and size along the epididymal duct. For example, principal cells in the initial segment are tall resulting in a duct with a small luminal diameter whereas in the cauda region, the principal cells are low columnar and luminal diameter is much larger (Fig. 1, 2). Through extensive analyses a much clearer picture is beginning to emerge regarding the function of each cell type within each epididymal region. Principal cells are known to actively secrete ions, organic solutes and proteins. They are involved in endocytosis and many receptors and transporters are localized to their apical and plasma membranes. Clear and narrow cells play a significant role in the acidification of the luminal fluid and also contain endocytotic machinery. Maintaining an acidic pH luminal fluid microenvironment is important for sperm maturation.

Basal cells express a number of antioxidant proteins and are thought to play a role in protection from oxidative stress and xenobiotics. Halo cells are a mix of T lymphocytes, monocytes and cytotoxic T-lymphocytes and may have a role in immune protection. The function of apical cells is unclear; however, there is evidence demonstrating that they endocytose material from the epididymal lumen. Surrounding the entire duct are differing layers of smooth muscle/myoid cells (Fig. 2) with the most numerous layers observed around the distal epididymis and vas deferens regions. Smooth muscle contractions aid the movement of spermatozoa and fluid along the epididymal duct.

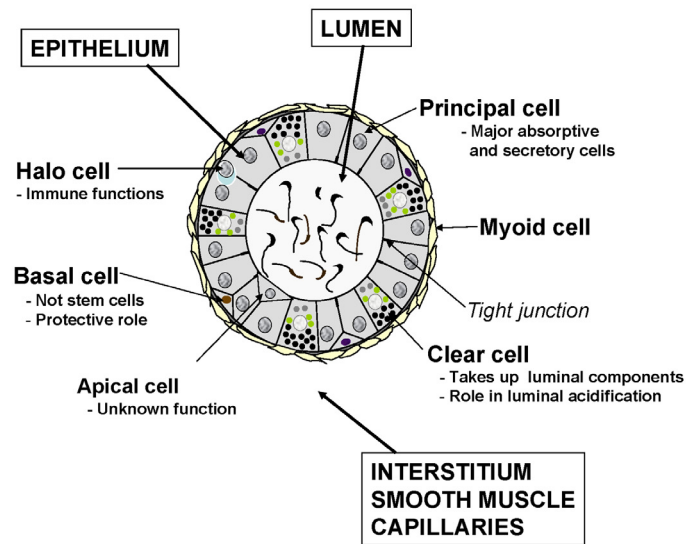


FIG. 2. Schematic organization of the major cell types in the epididymis as observed at the light microscope. The three epididymal compartments as well as the relative position and distribution of each of the main cell types are illustrated. The major function(s) associated with each cell type is also identified. Adapted from Robaire B, Hinton BT, Orgebin-Crist M-C. The Epididymis. In: Neill, JD, ed. *Physiology of Reproduction, Third Edition*. New York: Elsevier. 2006; 1071-1148.

### The blood-epididymis barrier

In view of there being a blood-testis barrier, it is not surprising to find a similar protective barrier throughout the epididymis. Physiological barriers perform several functions including providing a specialized luminal fluid microenvironment/milieu, protection against blood-born pathogens and

xenobiotics, as well as providing immune-privilege. Classically, physiological barriers have been thought as being only the tight junctions between cells. It is now clear that barrier function is a complex interaction between the permeability properties of the basolateral and apical membranes, e.g. presence of channels and transporters, the permeability of the tight junctions themselves, i.e. the paracellular route, and any immune protective mechanisms provided in the lumen and the interstitial space. The blood-epididymis barrier is highly dynamic and its properties constantly change from initial segment to vas deferens. From a clinical perspective, the blood-epididymis barrier is a formidable hurdle to overcome when designing potential male contraceptive agents. However, small molecular weight novel male contraceptive agents could be designed that would be specifically transported into the epididymal cells/lumen by transporters located on the basolateral and apical membranes.

### Animal models displaying epididymal and infertility phenotypes

Another challenge for investigators is to understand the role of secreted ions, organic solutes and proteins during sperm maturation. One approach addressing this challenge is to generate a series of gene null mutations in mice that display an epididymal phenotype and infertility. The best known of the null mutations is *Ros1* (*c-Ros*), an orphan tyrosine kinase receptor. Spermatozoa from these null animals display flagella angulation when exposed to the uterine, hypo-osmotic environment, rendering them incapable of reaching the egg for fertilization. Interestingly, the initial segment was found to be undeveloped in these animals, suggesting that the very proximal region of the epididymis is important for male fertility. Other murine models have also been found to display an angulated sperm defect and/or undeveloped initial segment, these include: *GPX5Tag2*, *XXSry*, “*viable motheaten*” (SHP-1 protein tyrosine phosphatase) null, Apolipoprotein E receptor 2 null, Acid sphingomyelinase null, *Herc4* null and *Foxi1* null. *Foxi1*, a transcription factor, is of particular interest because it is known to regulate the expression of vacuolar  $H^+$ -ATPase proton pump, carbonic dehydratase II and the chloride/bicarbonate transporter found in narrow and clear cells. This null model provides clear evidence for the importance of the luminal fluid microenvironment during sperm maturation, changing the pH of the epididymal luminal fluid microenvironment in these animals resulted in male infertility.

In summary, the epididymis promotes sperm maturation, facilitates the transport of spermatozoa along the duct, stores spermatozoa and protects

them from harmful substances. All of these functions are coordinated with remarkable precision to ensure production of fully viable spermatozoa.

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## What is the prostate and what are its functions?

G.S. Prins

### Male accessory sex glands

The prostate gland is one of the male accessory sex glands. To appreciate its function, one must understand the role of accessory sex glands and the production of semen. The accessory sex glands consist of the prostate gland, the paired seminal vesicles, located at the base of the bladder, and the Cowper's gland (bulbourethral glands) directly connected to the urethra. Semen consists of spermatozoa and the fluid bathing them produced by the testes and matured in the epididymis combined with seminal fluid produced by the accessory sex glands. The function of seminal fluid is twofold; 1) it serves as a buffered, nutrient transport medium for sperm as they are deposited in the female vagina, and 2) it retains sperm within the vagina for an optimum time period to permit proper activation of sperm capacity to fertilize and entry into the upper female genital tract.

It is generally accepted that seminal plasma substances are not essential for fertilization of the egg by mature sperm. This has been highlighted with successful *in vitro* fertilization using sperm surgically harvested from the epididymis or testes and thus never exposed to seminal fluid. Nonetheless, it is clear that seminal plasma enhances the *in vivo* fertilizing capacity of sperm. The slightly alkaline (7.2 - 7.8) pH of seminal plasma neutralizes the acidic vaginal environment. Seminal fructose provides energy for sperm, prostaglandins aid in smooth muscle contractions of the female genital tract and assist in sperm transport and specific proteins coat the sperm surface and prevent premature activation of factors necessary for egg penetration. Zinc and IgA act as bacteriostatic factors while anti-agglutination proteins prevent sperm cells from clumping together.

During emission and ejaculation, sperm move from their storage site in the epididymis through the vas deferens, propelled by peristaltic contractions of the vasa musculature. This is coordinated with contractions from the accessory sex glands and the combined contents – the semen – are expelled through the urethra. However, the accessory sex glands do not contract simultaneously but rather in a specific sequence. With adequate sexual stimulation, a small initial fraction from the Cowpers gland is released prior to ejaculation to lubricate the urethra. When ejaculation begins, a sperm-rich fraction is expelled containing sperm from the vas deferens along with prostatic secretions. This fraction is ~0.5 ml in volume or 25% of the ejaculate, the bulk of this volume coming from the prostate gland. The last and largest fraction of the ejaculate comes from the seminal vesicles and

varies between 1.0-2.5 ml or 75% of seminal volume. Spermatozoa and the epididymal fluid bathing them make up less than 10% of the volume of the ejaculate.

Soon after ejaculation, the semen coagulates, forming a gelatinous clot that restricts free movement of spermatozoa. Coagulation occurs by coagulating factors and unique enzymes produced by the seminal vesicles, similar to the thrombin coagulating system found in blood. After 15-30 minutes, the coagulated semen begins to liquefy as a result of proteolytic digestion by enzymes produced in the prostate gland. These prostatic enzymes include a chymotrypsin-like enzyme termed seminin, urokinase and distinct plasminogen activators. The liquefaction process permits the slow release of sperm from the coagulum, allowing them to be transported into the cervix and eventually upstream to the ovulated egg. Overall, the process of coagulation / liquefaction allows for appropriate exposure of the sperm cells to seminal fluid factors that stimulate motility and enhance fertilizing capacity and then permits an orderly entry of sperm cells into the upper female genital tract.

### Prostate gland anatomy

The prostate is a small walnut-sized gland that resides at the base of the bladder surrounding the urethra (Figure 1). In its center, the urethra makes a 70° turn at a site referred to as the verumontanum. The seminal vesicle ducts and vas deferens merge cranial to the prostate to form paired ejaculatory ducts that transverse the prostate gland and empty into the urethra at the level of the verumontanum. Below this site, 15-30 excretory ducts from the prostate enter the urethra and deposit prostatic secretions during ejaculation. This later region of the urethra is referred to as the prostatic urethra. Although the prostate gland is not lobular, there are distinctive regions or zones based on anatomic site, histologic appearance and propensity for disease. The central zone, lies between the ejaculatory ducts from the bladder base to the verumontanum and represents ~20% glandular volume. The peripheral zone surrounds the central zone and extends downward to the prostate apex, comprising 70% of prostatic volume. The prostatic ducts that empty into the prostatic urethra are within this peripheral zone. The transition zone (~10% volume) lies adjacent to the proximal urethra just above the verumontanum and is in contact with the central and peripheral zones at this site. The periurethral zone is comprised of tiny ductal branches within the proximal urethral wall adjacent to the preprostatic sphincter near the bladder. It is noteworthy that benign prostatic hyperplasia (BPH) develops in the periurethral and transition zones surrounding the proximal urethra whereas most prostate cancers develop within the peripheral zone.

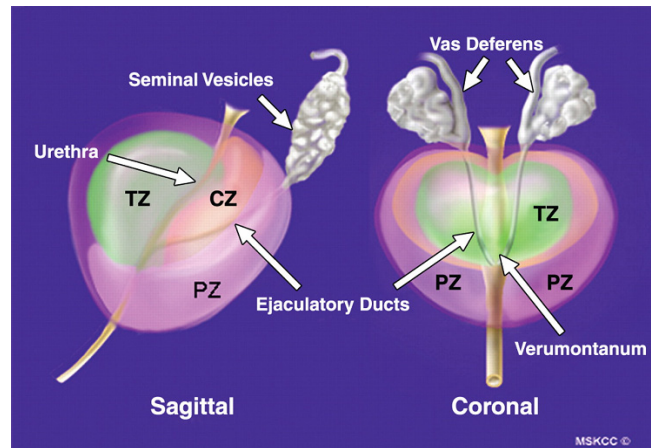


FIG. 1 Schematic representation of the prostate gland. PZ: Peripheral zone. TZ: Transition zone. CZ: Central zone. (Reprinted with permission from Currin S. et al, Am J Roentgenol 2007; 188: 13737).

### Prostate gland disease

The prostate gland is widely known for its propensity to develop diseases that interfere with quality of life and, in some cases, are fatal. This is unique among the male accessory sex glands and may be related to its embryologic origin from the endodermal urogenital sinus in contrast to the other accessory sex glands derived from the mesodermal Wolffian ducts. Three major prostatic diseases, in decreasing order of frequency, are prostatitis, BPH and prostate cancer. These are diseases of the aging male, most often appearing after the age of 50. Although a brief overview of the latter two conditions is presented below, each will be discussed in greater detail in chapters of their own. Prostatitis, an inflammatory condition of the prostate gland, can be both acute and chronic and affects ~50% of men during their lifetime. Interestingly, less than 10% of cases are due to bacterial infections and the etiology of the majority of prostatitis is unknown. The primary symptom of this disease is pelvic pain and treatments may include antibiotics, alpha-blockers, anti-inflammatory drugs, muscle relaxants, heat therapy or repetitive prostatic massage.

Benign prostatic hyperplasia or BPH is a noncancerous enlargement of the prostate gland due to its continued growth with aging. BPH occurs in 40-50% of men over 50 years of age and reaches 80% over 80 years. Since the prostate surrounds the urethra, BPH can decrease urine flow rate by increasing the flow-resistance within the urethra and may lead to various

lower urinary tract symptoms such as urinary frequency and urinary retention. Treatments are necessary in ~25% of patients and include medical management with alpha-blockers or 5 $\alpha$ -reductase inhibitors, stents and surgery.

Prostate cancer is the most common noncutaneous cancer in American men and the second leading cause of cancer-related deaths in the United States. Risk factors include aging, family history and ethnicity with African-American men having a 2:1 incidence ratio compared to Caucasians and Asian men having the lowest incidence world-wide. Prostate cancer has a life-time risk of 1 in 6 men in the USA with an incidence of 1 in 45 between 40-60 years and 1 in 7 between 60-80 years. While up to 50% of cancers remain latent, i.e. do not progress beyond the prostate gland, the remainder progress at variable rates which can lead to distant metastasis and death. At present, it is not possible to distinguish between these cancer types during early stage disease making treatment choices difficult. Early detection of prostate cancer has increased due to monitoring of prostate specific antigen (PSA) levels in the blood which can detect ~70-80% of cancers. Treatments include watchful waiting, surgery and radiation for early stage disease (i.e. confined to prostate) and chemotherapy and androgen deprivation for cancer at later stages. Like the prostate gland itself, prostate cancer initially depends on androgens to grow. Unfortunately, as the disease progresses, it becomes independent of androgens with no curative treatments available. Side effects of surgical and radiation therapy can include incontinence and impotence. Side effects associated with hormone therapy include infertility and muscle and bone wasting. Treatments focused on new drugs and vaccines are currently under development.

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## How does semen analysis assist in understanding the reproductive status of the male?

C. Wang

### What composes semen?

Semen consists of spermatozoa mixed with secretions from the testis (Chapter 6) and epididymis (Chapter 10) which during ejaculation are diluted with secretions from the prostate gland (Chapter 11) and seminal vesicles. The structure of the human spermatozoon is shown in Fig.1. The spermatozoon consists of the head which has the nucleus (chromatin containing the genetic material) covered by the acrosome; the mid-piece with the mitochondrion; and the tail (flagellum or principal piece) with microtubules forming the axoneme enclosed by the fibrous sheath which is essential for progressive forward movement of the spermatozoa. The shape and size of the sperm differ in different species as shown in Fig. 2.

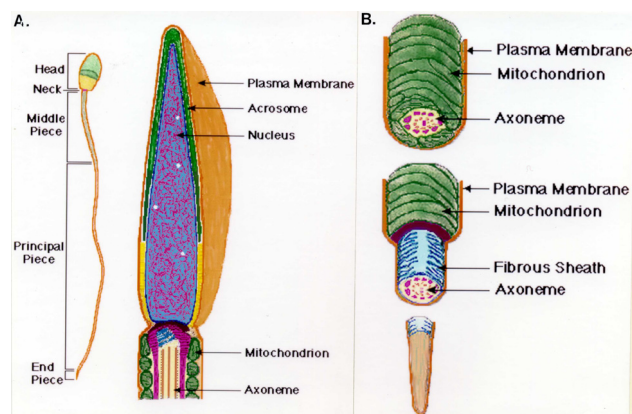


FIG. 1. A. Major elements of a human spermatozoon that are common to mammalian species. B. Middle piece (top), principal piece (middle), and end piece (bottom) of a spermatozoon viewed in cross-section.

The seminal fluid is made up a mixture of secretions from the testis, epididymis, prostate and seminal vesicles and the contribution from each of these glands varies by species and depends on the interval of abstinence and the method used to obtain the semen samples. In men, there are some reports indicating that semen samples collected by masturbation may be of a

lower quality than those recovered during sexual intercourse. Because the volume of the seminal fluid may be quite variable, it has been suggested that the total number of spermatozoa in the ejaculate may be a more important parameter than the sperm concentration in the ejaculate.

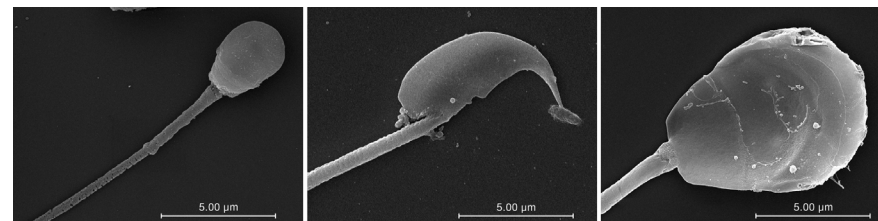


FIG. 2. Scanning electron micrographs of human (left), mouse (middle), and guinea pig (right). Spermatozoa. Courtesy of G. Hunnicutt, The Population Council.

### How is semen analyzed?

The World Health Organization (WHO, 2009) has developed a manual to provide a guide regarding acceptable approaches to semen analysis in man. This section will focus on these norms. Most of the techniques can be applied to other species. In rodents, the ejaculated semen forms a coagulum and does not liquefy, thus sperm counts are done by examining the spermatozoa in the cauda (tail region) of the epididymis. In rabbits, semen can be collected by an artificial vagina. In other species such as cattle, horse, and many of the zoo animals, semen is collected by electro-ejaculation. In monkeys, stimulation using a penile cuff appears to be more efficient than electro-ejaculation. In men, semen is obtained for analyses by masturbation. Collection using a condom during intercourse is not recommended because of presence of spermicidal or interfering agents in some condoms and loss of part of the ejaculate during intercourse. The duration of abstinence is noted because short periods of abstinence are associated with low semen volumes and sperm numbers. The sample is allowed to liquefy (by proteases present in the seminal fluid) and the following are usually assessed:

- volume, viscosity, appearance and pH of the seminal fluid
- sperm aggregation and presence of other cells (light microscopy)
- sperm motility using light microscopy
- sperm concentration using a hemocytometer (counting chamber) under the microscope
- sperm vitality after staining

- sperm morphology after preparation of smear on a slide
- other special tests as indicated.

There are fluctuations in semen parameters from day to day in men and usually at least two semen samples are required to diagnose that the semen quality is below the reference range of adult men. A recent study of over 4500 samples from men living in four continents gives the reference range of adult men. The lower reference limits (5<sup>th</sup> percentile) for semen parameters in “fertile” adult men are:

- semen volume 1.5 mL
- total sperm number per ejaculate 39 million
- sperm concentration 15 million/mL
- sperm progressive motility 32%, total sperm motility 40%
- sperm vitality 58 % alive
- spermatozoa with normal morphology (using strict criteria that exclude any spermatozoa with even a mild abnormality) 3 %.

Other tests to assess sperm function may include the ability of spermatozoa to interact and penetrate human cervical mucus and sperm antibody tests when sperm aggregates are noted. Special staining may reveal white blood cells in semen samples; this is suggestive of an infection. Semen biochemistry is indicated only when accessory organs problems are suspected, e.g., semen fructose is low in men with congenital absence of the vas deference and seminal vesicles. Assessment of sperm function may include tests of sperm chromatin (sperm damage, Chapter 13); the ability of the sperm to swell under hypo-osmotic conditions (test of sperm membrane integrity); the ability of the sperm head to lose the acrosome cap upon stimulation (acrosome reaction, a pre-requisite for fertilization); whether spermatozoa can bind human zona pellucida (testing the ability of spermatozoa to bind to zona) and penetrate a zona free hamster egg (the ability to penetrate the egg membrane and fertilize an oocyte). Computer assisted assessment of sperm motility characteristics has not proven to be very useful for clinical diagnosis but is frequently used in research and epidemiological studies.

#### **How does semen analysis help in assessment of male reproductive disorders?**

Semen analysis is usually the cornerstone of the assessment of the reproductive capacity of male animals. Rodent semen does not liquefy and cannot be used for analyses. For animal breeders, semen analyses provide a

guide to determine which animal should serve as a sire for multiple generations of animals. Semen analyses are used by reproductive toxicologists to study effect of exposure to environment and toxicants on male reproductive function. Suppression of the number or motility of spermatozoa in the ejaculate to very low levels is the goal of male contraceptive development. However, in clinical medicine semen analyses is used primarily for the diagnosis and treatment of male infertility. The diagnosis for male infertility is dependent, in large part, on the analysis of semen samples. Most men diagnosed with have defects in spermatogenesis resulting in low sperm concentration (oligozoospermia), which is generally associated with poor sperm motility (asthenozoospermia) and increased abnormal sperm morphology (teratozoospermia). Thus when semen analyses showed grossly low values male factor infertility is diagnosed. The concentration and the quality of sperm parameters guide the clinician to determine the appropriate treatment and estimation of the prognosis.

How useful is semen analyses in predicting fertility potential in men? This was examined in a prospective study relating semen quality to probability of conception in 430 couples with first-time pregnancies. This study showed that increasing sperm concentration up to 40 million/mL were associated with increasing conception probability. The proportion of sperm with normal morphology was strongly related with likelihood of pregnancy. In another study, the time to pregnancy in 942 couples increased with sperm concentration up to 55 million/mL and percent spermatozoa with normal morphology to 19 %. Thus from these prospective large clinical studies, sperm concentration, total sperm count and the proportion of sperm with normal morphology are important predictors of male fertility up to certain thresholds. Increasing these parameters to beyond these thresholds did not appear to increase the conception probability. How useful are additional functional tests in predicting fertility has not been studied in prospective large scale clinical studies.

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## How is sperm chromatin structure quality assessed? What is the value of doing such assessments?

### *Range of methods that assess sperm chromatin quality*

B. Robaire

International standards to assess semen parameters have been developed and updated over several decades by the World Health Organization (WHO), with the most recent revision appearing in 2010. As discussed in Chapter 12, these parameters are useful in population studies. At the extremes of parameters such as sperm number, concentration or sperm motility, it is clear that there is a strong correlation with fertility outcome. However, the semen samples of many individuals fall well below the WHO standards, yet they can father children and many men with samples that meet the WHO standard are not fertile. A very large proportion of unexplained (idiopathic) infertility may be due to factors other than those classically measured using the methods described in the WHO manual.

The parameters used in standard semen analysis are not designed to assess the “quality” of the spermatozoa, other than their ability to swim and “appear” normal. But what matters for successful fertility is not just getting to the oocyte but also delivering a nucleus (chromatin package) that is not damaged and capable of supporting the development of normal, healthy progeny. There are many animal studies showing that spermatozoa that are damaged due to exposure to chemicals, drugs or radiation, but that otherwise appear normal, do not have a disadvantage in fertilizing eggs and can deliver their damaged chromatin to the oocyte. Therefore, assessing the quality of the chromatin of spermatozoa has become of growing interest in the last two decades.

There are many aspects of the components of the nuclei of spermatozoa that can be assessed. These include not only the integrity of the DNA (single or double strand breaks, cross-links), but also the way it may be modified (DNA methylation) or how it is packaged and associated with the nuclear matrix. In addition to DNA, sperm nuclei contain small non-coding RNAs (pi and micro RNAs) and proteins (protamines, some histones, and nuclear matrix proteins).

Starting more than forty years ago, techniques such as alkaline elution were developed to assess aspects of chromatin quality such as DNA breaks and cross-links. Some of these assays, such as the nuclear decondensation assays (either in vitro or in vivo) or the DNA template function assay, are

used less frequently now because of the development of more rapid and efficient new methods. During the past twenty years a number of these newer methods have been developed using light, fluorescence and confocal microscopy, flow cytometry, real time PCR and high throughput screening. There are a number of ways of grouping these many assays. We will provide here a highlight of some of the assays that are more commonly used or under active development.

Assays used to evaluate genomic integrity include:

- **Comet Assay.** This electrophoresis-based assay evaluates DNA strand breaks in a single cell. Small, broken pieces of DNA migrate away from the DNA core of the cell, creating the tail of the comet figure. The percentage of the DNA in the tail of the comet and the tail length are measured using specific image analysis software. Depending on whether it is run at basic or neutral pH, the Comet Assay will assess single or double strand breaks, respectively. This is a very sensitive assay, but it is time consuming and variable among labs.
- **TUNEL Assay.** The quantity of DNA 3'-OH ends can be assessed using the terminal deoxytransferase mediated dUTP nick end-labeling (TUNEL) assay in which the terminal deoxytransferase enzyme incorporates a fluorescent UTP at 3'-OH free ends; fluorescence increases proportionally with the number of DNA strand breaks. This assay can be run as either a slide-based or flow-cytometry assay.
- **Gene-Specific DNA Damage and Repair Assay.** Using quantitative PCR, the presence of single/double strand DNA breaks and/or the extent of bulky DNA adducts are assessed. This assay depends on the principle that increased bulky adduct formation or DNA breaks prevent the polymerase from completing amplification of the complementary strands, thus causing less PCR product to be amplified. An advantage of this assay is that it requires very small (nanogram) amounts of DNA, but it is time consuming and can be difficult to do accurately.
- **Fluorescence in situ hybridization (FISH) Assay.** By using probes specific to individual chromosomes, the frequency of chromosomal aneuploidy (duplication or deletion of whole or part of chromosomes) can be assessed in spermatozoa after proper decondensation. Multiple probes are available for human spermatozoal chromosomes, but until this method is automated, having to score several thousand of sperm manually for accurate result render this method still primarily a research tool.

Assays used to evaluate sperm chromatin conformation and proteins include:

- *Acridine Orange or SCSA® Assay.* A commonly used flow-cytometry based assay wherein the susceptibility of sperm chromatin to denaturation in acid and detergent is determined by using acridine orange, a dye that binds to double- or single-stranded DNA, giving a green or red fluorescence, respectively. After denaturation, measurement of fluorescence at both wavelengths assesses the percentage of fragmented DNA (DNA fragmentation index: DFI). This assay may be run as a “slide assay”, where the color reading is made on microscope slides, or as a FACS assay, where large numbers of sperm can be analyzed rapidly.
- *CMA3 Fluorometric Assay.* This assay has been developed to indirectly measure the amounts of protamine present. The dye CMA3 binds to sites in DNA where protamines would normally bind. This assay was first developed as a slide based assay but is now available as a FACS assay.
- *Monobromobimane (mBB) Thiol Labeling Assay.* The objective of this assay is to determine the amount of free sulphhydryl groups in spermatozoa; less mature sperm have more free sulphhydryls whereas more mature sperm have fewer. Thiol labeling is done to quantify the total thiol, free thiol and disulfide levels in the nuclei of spermatozoa. As for the two assays above, this may be run as a slide-based or a FACS assay.
- *Sperm Chromatin Dispersion Test.* In this assay, the extent of halo formation of single spermatozoa, representative of the decondensation of their nuclei, is determined for cells that are embedded in agarose, lysed and acid treated. The larger the halo, the greater the extent of DNA breaks. Even though the results obtained using this technique have been correlated with double strand breaks, it is not a direct measure of DNA breaks but a reflection of the overall chromatin structure.
- *Proteomic Analysis.* The specific chromatin structure of the sperm is essential for proper fertility and is, in part, due to the proteins that are bound to the DNA, including the protamines, histones and components of the nuclear matrix. With the recent advances in proteomics technology, using 1 or 2 dimensional SDS polyacrylamide gels coupled with mass spectrometry, various components of the sperm are being identified. This, in turn, increases our knowledge of the sperm chromatin structure composition, but also opens the possibility of new criteria to be looked at when assessing sperm quality.

Assays used to evaluate the epigenetic status of spermatozoa include:

- *DNA Methylation.* DNA methylation of cytosine residues is one of the major epigenetic marks established during spermatogenesis. Methods for assessing changes in methylation at specific sites are well established, but several methods are under rapid development that will allow accurate assessment of changes in DNA methylation throughout the entire sperm genome.
- *Histone Modifications.* Most histones are removed during spermatogenesis but a significant proportion, up to 15% in man, remains in mature sperm. The role of these histones is still under debate, but recent studies indicate that they mark genes that are activated early after fertilization. Alterations in either the amount and distribution of histones or their numerous marks, e.g., acetylation, methylation, sumoylation, may affect the functions of spermatozoa.
- *Small Non-coding RNAs.* The third major pillar of epigenetic regulation is the expression and function of small non-coding RNAs. The exact functions of the various RNAs (mRNAs, piRNAs, antisense and miRNAs) are not known, but they clearly play an essential role in spermatogenesis and potentially the post-fertilization embryo. It is likely that their relationship to the quality of spermatozoa will become evident as our knowledge grows about these molecules.

No consensus has yet emerged regarding the value of any one or group of tests in assessing the fertility of an individual (Chapters 12,19). However, the growing power, precision and accuracy of newer tests, based on the molecular structure of sperm nuclei, makes it likely that new clinically useful tools to accurately assess the quality of spermatozoa will emerge.

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## **What is sperm banking? When and how is it (or should it be) used in humans? Animals?**

### *Sperm banking, consequences of its use in animal and clinical practice*

S. Rothmann

#### **Definition and history**

Sperm banking, more formally referred to as sperm cryopreservation, is a process intended to preserve sperm function by freezing and storage at ultra-low temperature. Upon thawing, sperm are introduced into a suitable recipient female by insemination into either the endocervical canal or the intrauterine cavity, or are used to inseminate oocytes during in vitro fertilization. Sperm freezing originated in the late eighteenth century, but the development of many sperm banking applications began after 1950 following the discoveries that glycerol can act as a cryoprotectant and ultra low temperature liquid gases, especially liquid nitrogen, for freezing and storage.

The benefits of sperm cryopreservation include:

- almost indefinite storage (at least multiple decades), allowing preservation of fertility potential that would be lost due to onset of disease, infertility, gonadotoxic events or death
- ability to "quarantine" the sperm while the human or animal donor can be tested for semen-borne infections or genetic anomalies
- acquisition of sperm in advance for subsequent use
- shipping to locations distant from the donor

#### **The process of sperm cryopreservation**

In spite of the important uses of cryopreserved sperm, little is known about the physical and biochemical events that occur during sperm freezing, storage, and thawing, or about how to adequately detect cryogenic damage. Sperm from most species survive current cryopreservation protocols very poorly, and best efforts usually result in recovery of only about half of the original sperm motility. Sperm function is also impaired, as manifested after thawing by shortened longevity, poor cervical mucus penetration and reduced membrane stability.

The goals of sperm cryopreservation methods are to prevent lethal intracellular ice crystal formation, to control wide fluctuations in cell volume and to reduce membrane damage that accompanies temperature-induced

phase changes. The biochemically and physically diverse compartments of the sperm cell (acrosome, nucleus, mitochondrial-flagellar network) complicate the process, since they may respond quite differently to freezing and thawing. The sperm also are subject to damaging oxygen radical exposure during their transit through wide temperature changes. Attempts to maximize post-thaw survival have led to the development of sperm cell diluents (semen extenders), cryoprotectants, and various rates of temperature change to control alterations in extracellular and intracellular solvents and solutes.

In a typical slow-freeze cryopreservation protocol, the semen is mixed with a buffered diluent or extender that contains lipids (often egg yolk), a penetrating cryoprotectant such as glycerol and an energy source such as fructose or glucose. After dilution, the sperm initially undergo rapid shrinkage as intracellular water leaves the cell, and then slowly return to their original volume as the glycerol enters. Rapid cooling is initiated at a rate of about -20°C per minute. Extracellular formation of ice crystals begins and, as water freezes, the solutes present in the liquid phase surrounding the sperm rapidly become concentrated. Glycerol lowers the intracellular water freezing point, thus the cells remain unfrozen and become supercooled well below their actual freezing point. In response to high extracellular solute concentration and the osmotic tendency of supercooled intracellular water to leave the cells, sperm undergo a second volume adjustment as water moves outward, and the cells become dehydrated. When extracellular water freezes and therefore solidifies, an exothermic reaction known as the "heat of fusion" occurs, which can cause serious disruption of the cells, unless deliberately reduced by controlled cooling of the environment. Upon reaching the temperature of liquid nitrogen, -196°C, the sperm are placed in liquid nitrogen or vapor phase storage, where they are presumed to reside in a quiescent state of minimal molecular motion indefinitely.

During thawing, the sperm are subjected to similar rapid and dramatic changes in cell volume and membrane permeability. When the extracellular ice melts and becomes liquid, solute concentrations are rapidly diluted and water rushes into the sperm. As the temperature rises, and as glycerol leaves the cells, the sperm cell volume continues to expand. In order for sperm function to be restored, the surface area and volume must return to normal, the membrane proteins and lipids must redistribute to restore molecular structure and mobility and bioenergetic demands must be met.

For maximum functional recovery to take place, both the freezing and the thawing protocols must be optimized, a very difficult task given the paucity of data available about these processes. Recent research has focused on mechanisms of freeze-thaw damage and alternate freezing methods such as vitrification, ultra-rapid freezing without a cryoprotectant.

## **Human clinical applications of sperm banking**

Clinical applications of sperm banking usually are divided into donor sperm cryopreservation or client depositor (autologous) sperm cryopreservation. In either case, sperm are obtained most commonly by masturbation, but alternatively can be retrieved by electro-ejaculation or through surgical procedures such as epididymal aspiration, testicular aspiration or extraction. In the USA, the Food and Drug Administration (FDA) regulates human sperm banking under Human Cells, Tissues, and Cellular and Tissue Based-Products (HCT/PS) Regulation. Under the authority of Rule 1271, sperm banks must register with the FDA, adhere to guidelines for donor screening and follow standards for processing, storage and distribution. The American Association of Tissue Banks (AATB) has standards for both donor and client depositor sperm banking, and accredits banks by inspection. Several states require separate licensure with inspection. Many other countries also have regulations about sperm banking.

The most commonly used donor sperm banking depends on anonymous donors for infertility caused by absent or defective sperm. In a 1987 survey, the United States Office of Technology Assessment estimated that 30,000 births resulted from artificial insemination of donor sperm, with approximately 11,000 physicians providing the treatment to about 86,000 women. Although fertility specialists believe that the practice has decreased as assisted reproductive technologies improve the ability to achieve pregnancies with low numbers of sperm, the demand for fertile and safe sperm remains high, especially among single women and women only couples.

The practice of using cryopreserved sperm for donor insemination arose in the mid-1980s after the emergence of human immunodeficiency virus (HIV). The incubation period before the disease could be detected made screening at the time of collection unreliable. The recognition that this problem also exists for most other semen-borne infectious diseases led to the use of a cryopreservation “quarantine” to improve safety. This practice permits repeated examination of the donor for disease exposure over weeks, months or years before the sperm are used. After a minimum of 180 days quarantine, the sperm can be used as long as the donor retesting is negative for infectious disease. As nucleic acid testing for diseases improves and replaces traditional serologic testing, reduction of the quarantine period may be possible.

The ability to store sperm from men with many different phenotypes and genotypes increases the selection that patients have in choosing a donor and reduces excessive use of a donor within a limited geographic area. Population statistics can allow determination of the number of pregnancies that can be achieved without increasing the risk of consanguinity in future

generations. Generally, sperm from a single individual are used to achieve no more than 10-15 pregnancies in a medium-sized city (500,000 to 1,000,000 inhabitants) in the United States. In other countries where ethnic diversity and ethnic intermarriage are not as common, the number could be smaller, but depends in any case on the live birthrate and number of inhabitants.

Usually, sperm banks attempt to package donor sperm in plastic vials or straws containing at least 10 million motile sperm post-thaw, which has been suggested as the minimum adequate insemination dose. Since frozen-thawed sperm have shorter longevity than fresh sperm, the route and timing of insemination is critically important in achieving a successful pregnancy. Using qualitative urinary luteinizing hormone (LH) measurement to predict ovulation, and one or two intrauterine inseminations within 20 to 40 hours after the LH surge, approximately 70% of patients who elect donor sperm insemination conceive, most within six insemination cycles.

Client depositor sperm banking, where a man preserves his own sperm for later use, is useful in the following situations:

- 1) Medical disorders that inherently, or through the treatment used to cure or stabilize the disease, can impair fertility by causing decreased sperm count and function, early fetal loss, genetic mutation, or impotence. Common examples include cancers, Hodgkin and other lymphomas, leukemia and myelodysplastic disorders, nephrotic syndrome, diabetes and multiple sclerosis. The new medical practice of oncofertility promotes fertility preservation as an essential part of oncology treatment, and hopefully will increase the utilization of sperm banking.
- 2) Prior to elective sterilization or exposure to hazardous environments. Occupational exposure to radiation, pesticides, and chemicals can affect sperm function or genetic integrity. Men engaging in military operations where risks of death or exposure to sperm toxicants exist are also candidates for sperm storage.
- 3) Before participating in fertility treatments that require semen collection at a specific time. For men who develop anxiety-related impotency or emission failure, sperm banking ensures that treatment cycles can proceed as planned. Patients whose occupation require unscheduled travel also use sperm banking to reduce the risk of cancelled treatment cycles.

The relatively few referrals for client depositor sperm banking may be linked to concerns about the quality and utility of sperm in men with systemic diseases. Although sperm count, motility and physiology may be impaired before treatment is initiated, the technological advances in assisted

reproduction, such as direct sperm injection into the ooplasm (intracytoplasmic sperm injection or ICSI), often can overcome many abnormalities. Having many sperm stored is definitely an advantage since it may reduce the need for in vitro fertilization or increase the chance for a successful pregnancy by allowing multiple cycles of fertility treatments. However, the desire to bank multiple ejaculates, that will require adequate abstinence period between sperm banking, must be balanced against the urgency of treatment initiation. Given the increasing number of reproductive centers and laboratories that can perform sperm banking, all men who might have compromised fertility as a consequence of their disease or its treatment should be offered the opportunity to cryopreserve sperm and, whenever possible, be given the time to store as many sperm samples as possible.

### **Sperm banking in animals**

Sperm cryopreservation has important uses in the livestock industry, especially in the breeding of cattle, pigs, sheep and poultry, and in animal husbandry for domesticated animals such as horses, cats and dogs. Sperm from genetically desirable or "prized" animals can be used to inseminate many females to increase the number of offspring with the desired characteristics. The ability to easily transport sperm has permitted the improvement of existing herds or the establishment of new herds in regions of the world needing development of native food sources. Sperm banking has also become an important way to perpetuate endangered or exotic species in the wild and in zoological parks.

The ability to use sperm banking to preserve important research animal strains has been appreciated recently. Sperm cryopreservation can reduce the extraordinary cost of maintaining genetic lines that would otherwise be preserved by continual breeding of the animals. It also increases the accessibility of various strains to researchers since frozen sperm are easier to transport than live animals. Sperm banking reduces the risk of losing a valuable genetic line through catastrophic accident, impaired reproductive efficiency, genetic drift or disease. Because the millions of sperm normally present in a single ejaculate also represent millions of meiotic recombination events, cryopreserved sperm can be stored for future studies of gene recombination frequency and mapping of genetic loci when new DNA probes become available.

### **Summary and opportunities**

Research efforts to improve sperm banking techniques and post-thaw

survival have intensified in the past few decades and have been renewed by the emergence of oncofertility as a medical specialty. As protocols improve, the success of cryopreserved sperm applications will undoubtedly increase. Numerous sperm banking career opportunities exist for basic and applied research, as well as for clinicians in both human and animal applications from the laboratory to the bedside or "barnside".

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## How does the sperm make its way to the egg and how does fertilization take place?

### *Capacitation, acrosome exocytosis, steps of sperm-egg interaction, egg activation*

J.P. Evans and J.L. Bailey

Fertilization is a complex, multi-step process. This fascinating biological event actually begins with both sperm and eggs undergoing a series of preparatory steps to make fertilization possible (Fig. 1). At ovulation, the egg (or multiple eggs, depending on the species) leaves the ovary for the oviduct. Concurrently, the egg progresses through meiosis, known as "oocyte maturation" or "meiotic maturation," from prophase I, where it has been arrested since before birth, to an arrest in metaphase II in most species. Sperm are morphologically mature upon leaving the testis, but they must undergo several steps to acquire fertilization competence. During epididymal maturation (Chapter 10), sperm develop the ability to (1) be motile and (2) undergo capacitation (addressed below), largely via reorganization of the sperm surface, that is thought to be important for the sperm's fertilizing capability. Next, upon ejaculation, the sperm are mixed with seminal fluid, which includes buffering components that allow sperm survival in the acidic environment of the vagina. Seminal fluid can also include proteins that attach to the sperm and later will mediate sperm binding to the oviduct (see below).

In the female tract, sperm undergo capacitation, defined as the physiological changes that confer the ability to fertilize an egg. It is notable that the discovery of methods to support sperm capacitation *in vitro* is one of the advances that made *in vitro* fertilization possible. Capacitation-associated changes include the loss of cholesterol from the sperm plasma membrane, activation of a soluble adenylate cyclase in the sperm, generation of the second messenger cyclic AMP, and activation of a number of protein kinases. Capacitated sperm are also now capable of undergoing acrosome exocytosis (addressed below). In many mammalian species, capacitation is associated with a change in the sperm's swimming pattern to a non-progressive, whiplash motion, which characterizes hyperactivated motility.

Sperm can be stored in the female reproductive tract by interacting with epithelial cells that line the oviduct. In most mammals, including humans, sperm can survive in the female tract for up to several days. Possible functions of sperm storage in the female tract include prevention of polyspermy (fertilization of the egg by more than one sperm), maintenance of sperm fertility and viability, and regulation of capacitation and hyperactivated

motility. Eventually, sperm travel to the site of fertilization, the ampulla region or the ampullary-isthmic junction of the oviduct, depending on the species. The transit of the sperm up the oviduct could be mediated by different factors, including contraction of the female tract to push sperm upward, sperm motility propelling migration up the tract, and/or sperm motility possibly directed toward a follicular or egg chemoattractant.

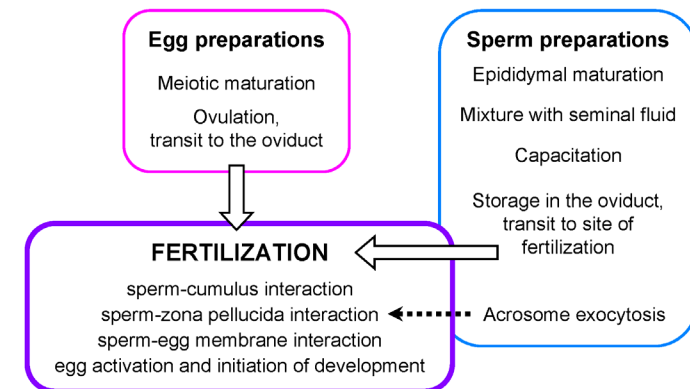


FIG. 1. Schematic diagram showing the steps discussed in this chapter, including the eggs preparatory steps (pink box), the sperm's preparatory steps (blue box), and the steps of fertilization itself (purple box). The dotted line indicates that acrosome exocytosis, the last step of the sperm's preparatory changes, occurs with sperm interaction with the egg's zona pellucida.

Fertilization itself occurs in several steps: (1) sperm-cumulus interactions, (2) sperm-zona pellucida interactions, (3) sperm-egg membrane interactions, and (4) egg activation and initiation of development (Figs. 1, 2). The ovulated egg is surrounded by cumulus (granulosa) cells embedded in an extracellular matrix made of hyaluronic acid. Sperm penetration through this matrix is mediated by the sperm's motile force and perhaps also facilitated by hyaluronidases on the sperm surface.

The sperm next interacts with the egg's coat, the zona pellucida (ZP), which is synthesized by the developing egg during oogenesis and is composed of three or four glycoproteins, depending on the species. The identity of the receptor on the sperm for the ZP has been debated for decades; it is likely not a single protein but a group of proteins. The ZP supports sperm binding in a species-selective manner and induces the sperm to undergo acrosome exocytosis. Also known as the acrosome

reaction, acrosome exocytosis is the liberation of the contents from the acrosome, the large secretory vesicle on the head of the sperm (Fig. 2). The release of these contents is accomplished by the formation of hundreds of fusion pores between the acrosome and the head plasma membrane. Acrosome exocytosis is linked with two critical changes in the sperm: 1) the acrosome releases enzymes to digest a hole in the ZP, and 2) upon completion of acrosome exocytosis, new surfaces of the sperm are exposed, which render the sperm capable of interacting with the egg plasma membrane. Thus, acrosome exocytosis is a necessary prerequisite for the next step of fertilization, gamete membrane interaction (see below). The signal triggering the sperm to undergo acrosome exocytosis is thought to be binding to a specific ZP component, although a recent hypothesis speculates that acrosome exocytosis in mouse sperm is induced by a mechanosensory signal as the sperm moves through the ZP matrix. Downstream from this initial trigger, calcium is a key second messenger that induces the sperm to undergo acrosome exocytosis.

Once the sperm has penetrated through the ZP, it reaches the egg plasma membrane. The interaction of the sperm with the egg plasma membrane is a multi-step process, beginning with adhesion of the sperm to the egg plasma membrane, bringing the membranes in close contact, and culminating in membrane fusion, which creates cytoplasmic continuity between the gametes (Fig. 2). The molecular basis of sperm-egg membrane interactions, like with sperm-ZP interactions, is not completely understood but this process likely involves multiple molecules. Upon formation of cytoplasmic continuity between the gametes, one of the intracellular components introduced into the egg from the sperm is a sperm-specific form of phospholipase C (PLC $\zeta$ ). This PLC generates inositol triphosphate (IP<sub>3</sub>) from phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). IP<sub>3</sub> in turn binds to IP<sub>3</sub> receptors on the egg's intracellular Ca<sup>2+</sup> stores, the endoplasmic reticulum. This causes release of Ca<sup>2+</sup> into the cytosol, activating several Ca<sup>2+</sup>-dependent enzymes. The activation of this signaling pathway induces the egg-to-embryo transition, also known as egg activation. The main events of egg activation are the establishment of blocks at the level of the ZP and/or the egg membrane to prevent polyspermic fertilization, the completion of meiosis (exit from metaphase II arrest) and progression to embryonic mitosis.

In sum, the sperm's mission to fertilize the egg and deliver the paternal genome to the future embryo involves a highly orchestrated series of steps that is initiated soon after leaving the testis and continues through the male and female reproductive tract, culminating with gamete fusion, egg activation, and merger of the maternal and paternal genomes.

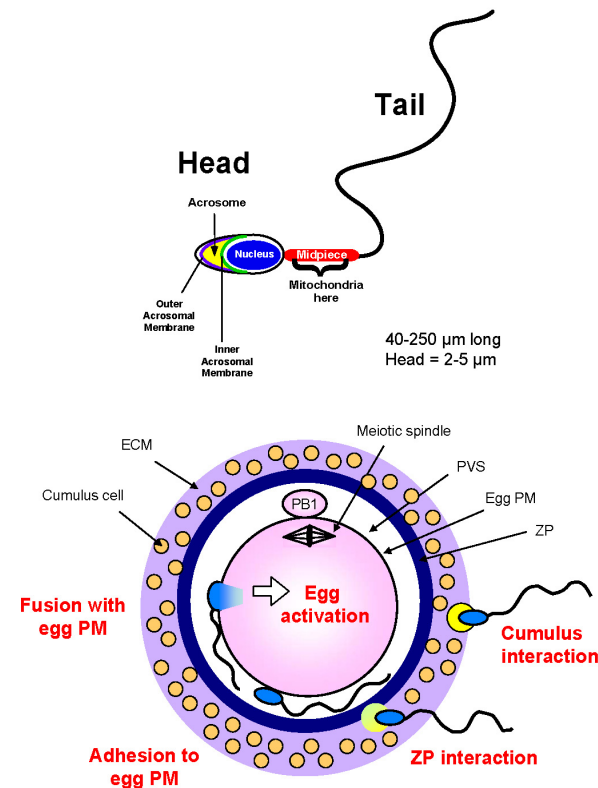


FIG. 2. *Upper panel:* Schematic diagram of a mammalian sperm, indicating the two main domains, the head and the tail. The flagellar tail contains microtubules, and includes the midpiece, which is where the mitochondria are located. The head includes the sperm chromatin in the nucleus (blue), and the acrosome (yellow). Acrosome exocytosis is achieved by the formation of fusion pores between the outer acrosomal membrane (purple) and plasma membrane (black). After acrosome exocytosis is complete, the inner acrosome membrane (green) is exposed on the surface of the sperm. *Lower panel:* Schematic diagram showing the structures of the mammalian egg (black text) and the steps of fertilization (red text). Abbreviations: ECM, extracellular matrix in which the cumulus cells are embedded; ZP, zona pellucida; PM, plasma membrane; PVS, perivitelline space, the space between the zona pellucida and the egg plasma membrane; PB1, first polar body, the product of cytokinesis resulting from the first meiotic division. The second meiotic division will not be completed until egg activation occurs.

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## What determines maleness?

### *Sex determination, testis formation and the development of the male phenotype*

D.W. Silversides and R.S. Viger

*To be (male) or not to be...  
That is the question (for andrology)*

Being male or female is a fundamental aspect of being human. Becoming male or female is a developmental story that unfolds at three levels (conception, sex determination and sex differentiation) and that involves the interplay of chromosomes, genes and hormones (Fig. 1). In the case of males, the result is an individual with an XY sex chromosome complement, having testicles and male external genitalia (penis and scrotum) and internal accessory sex organs (epididymis, vas deferens, seminal vesicle).

### Chromosomal sex

The definition of biological sex starts at conception. Although the human Y chromosome was reported in the 1920s, surprisingly it was not until 1959, through studies of Turner (XX) and Klinefelter (XXY) syndrome phenotypes, that the Y chromosome was associated with male sex determination. All human eggs contain a single X chromosome as part of their haploid genome. At the time of conception, if a Y chromosome bearing sperm cell unites with the egg, the resulting zygote will have an XY sex chromosome complement and will (normally) be male; if an X bearing sperm cell unites with the egg, the resulting zygote will have an XX sex chromosome complement and will be female. The correlation of the Y chromosome with maleness led to the hypothesis that it contained a “testis determining factor” (TDF); this realization led in turn to a 30 year quest to determine the molecular nature of this factor.

### The male phenotype and the importance of having testicles

Developmentally, the next important decisions for defining sex are the determination of gonadal sex (testes in males, ovaries in females), followed by the acquisition of the secondary sex phenotype (male or female). The go-

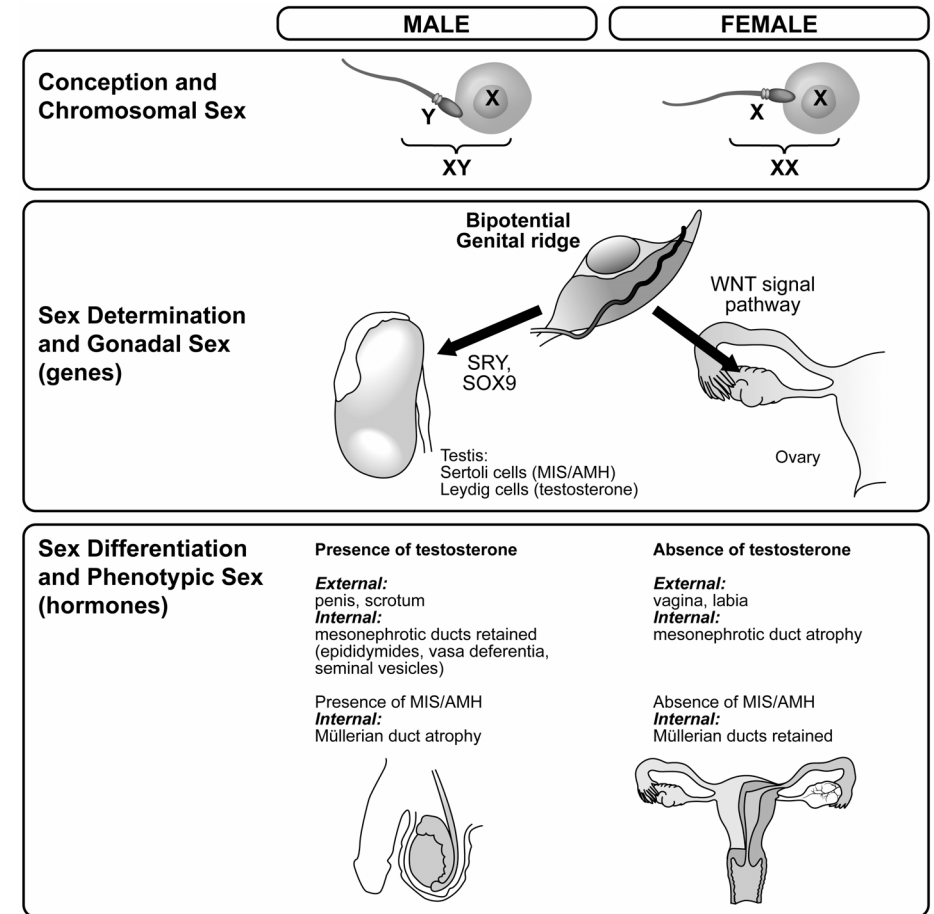


FIG. 1. Overview of biological sex in mammals.

nads develop from the paired genital ridges, found on the roof of the abdominal cavity of the fetus soon after gastrulation. The genital ridges initially have the capacity to form either testes or ovaries: they are bipotential. Historically it was known since the 1940s that the sex of the gonads is important for determining the phenotypic sex of the individual. More specifically, the presence of testes is required to insure a male phenotype, since removal of the genital ridge in an XY embryo was shown to result in a female phenotype. Hormones secreted by the developing testes are involved in these sex differentiation decisions. Sertoli cells of the developing testes



secrete the protein Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone or AMH), that causes the atrophy and loss of the paramesonephric (Müllerian) ducts in the XY fetus. Sertoli cells, along with germ cells, are now organized into cord like tubules that will become the seminiferous tubules. Leydig cells develop outside of these cord structures (in the interstitium). Leydig cells produce the steroid hormone testosterone. Testosterone and its metabolite dihydrotestosterone (DHT) cause the fetal external genitalia to develop into a penis and scrotum, and the internal mesonephric (Wolffian) ducts to be retained and develop into the epididymides, vasa deferentia and seminal vesicles. Testosterone produced by the male fetus will also masculinize the developing brain. In the XX fetus, the absence of testosterone (and presence of maternal and fetal estrogens) insures that the external genitalia develop as a vagina and labia and that internally, the mesonephric ducts atrophy. At the same time, the absence of MIS/AMH in the female allows the paramesonephric (Müllerian) ducts to be retained and develop into the oviducts (Fallopian tubes), uterus and cervix.

### **SRY and the molecular mechanism of testis determination**

Studies of rare deletions within the Y chromosome that resulted in discordance of chromosomal and phenotypic sex (i.e., XY females), along with the advent of molecular genetic techniques, culminated in 1990 with the cloning of a Y chromosome gene, named *SRY* (Sex determining region Y). When it was reported a year later that the introduction of *Sry* into an XX mouse genome resulted in a male phenotype, *SRY* was accepted to be the long sought-after TDF. How does it accomplish this role? *SRY* is expressed in pre-Sertoli cells of the developing XY genital ridge, just before the ridge starts its histological transformation to become a testicle. *SRY* was the first identified member of a larger family of DNA binding proteins, the SOX (SRY related HMG box) proteins. SOX proteins are important in making a number of key developmental decisions in animal embryos. Curiously, *SRY* is not a typical SOX gene: whereas other SOX genes are structurally well conserved between vertebrate and even invertebrate species, *SRY* is only found in placental mammals and has very poor sequence conservation between species. As a consequence of *SRY* expression, pre-Sertoli cells express another SOX gene named *SOX9*. In contrast to *SRY*, *SOX9* is highly conserved in vertebrates, and overexpression of *Sox9* in mice can force the development of testes in the XX fetus. *SOX9* will also turn on expression of the *MIS/AMH* gene. The picture now emerging for the molecular mechanism of mammalian sex determination is that the bipotential genital ridge, either XX or XY, is poised in a delicate balance between two competing developmental pathways, the male pathway dominated by *SOX9* expression,

and the female pathway which responds to the extracellular signal molecule WNT4. In the normal XY genital ridge, the presence and expression of the *SRY* gene in pre-Sertoli cells tips the balance towards increased *SOX9* expression and the male pathway—genital ridge development proceeds in the direction of testis formation. In the absence of *SRY*, WNT signaling dominates in cells of the genital ridge, favoring the development of the ovary.

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## Are there specific genetic defects affecting the male reproductive tract? What are the underlying mechanisms?

*Turner's and Klinefelter's syndromes, genetic loci, GnRH/LH/FSH, steroid biosynthesis and androgen insensitivity*

T.R. Brown

Normal development and function of the male reproductive tract begins in the zygote where the X and Y chromosomes establish the genetic sex and direct the sequential evolution of gonadal and phenotypic sex in the fetus (Chapter 16). Under normal circumstances, gonadal and phenotypic sex agrees with chromosomal sex; however, abnormalities of sexual phenotype and function can arise from various defects during development. Beyond the sex chromosomes, disorders of male sex differentiation, development and function can result from single gene mutations. The analysis of these disorders has been highly informative in defining the genetic and molecular determinants of sex development and reproductive function (Fig. 1).

### Gonadal disorders

#### *True hermaphroditism*

The clinical designation of true hermaphroditism is predicated upon the presence of both testicular and ovarian tissue. Subjects have a testis or ovary present in combination with a contralateral ovotestis containing both ovarian and testicular tissue (50%), or have a testis on one side and ovary on the other (30%), or bilateral ovotestes, or even bilateral ovary and testis combinations (20%). Functional oocytes may be present within the ovarian tissue whereas spermatogenesis is impaired within the undescended testicular tissue. The extent of functional testicular tissue will determine the internal duct structures. Secretion of Mullerian inhibiting substance (MIS) by Sertoli cells causes variable degrees of bilateral Mullerian duct regression, whereas local secretion of testosterone by Leydig cells is necessary for ipsilateral development of the Wolffian ducts. If a uterus is present on one side, an associated fallopian tube is often present. The external genitalia are usually ambiguous, although relatively normal male or female phenotypic appearance is possible. Hypospadias (abnormally placed urinary opening), cryptorchidism (undescended testis) or an inguinal hernia containing a gonad or Mullerian remnant may also be present. A majority of true hermaphrodites are raised as males due to the external appearance of

the genitalia, even though over 50% of subjects have a 46XX karyotype. Other karyotypes, such as 46XY, 46XX/XY chimerism or various forms of mosaicism may be present. Many 46XX true hermaphrodites do not possess the SRY gene suggesting that the etiology in these subjects differs from that of 46XX males who have a translocation of the Y chromosomal SRY (sex determining region of the Y chromosome) gene locus.

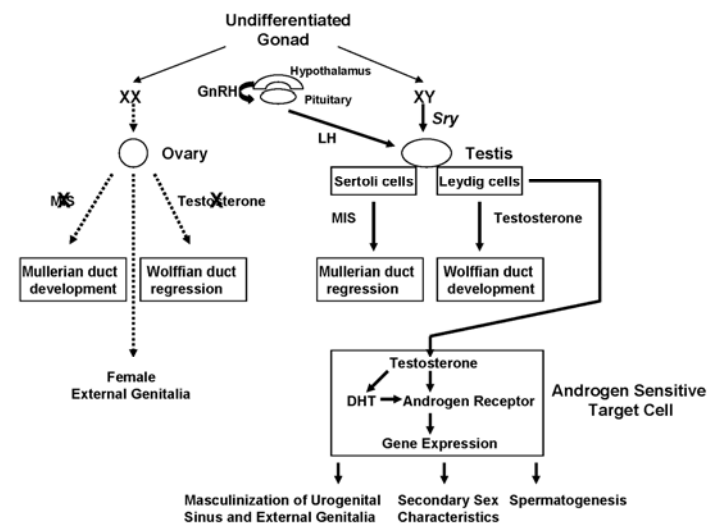


FIG. 1. Determinants of human sex differentiation and development. The undifferentiated gonad in the fetus develops into a testis when the chromosomal sex is XY due to the presence of the sex determining region of the Y chromosome, Sry, gene. If the chromosomal sex is XX, the undifferentiated gonad evolves into the ovary. Initially under the control of placental hCG, fetal testicular Leydig cells synthesize and secrete testosterone necessary for development of the Wolffian ducts. Sertoli cells in the fetal testis produce Mullerian Inhibiting Substance (MIS) which causes regression of the Mullerian ducts in the male. Subsequent to placental hCG, GnRH is synthesized and secreted by the hypothalamus to stimulate the synthesis and secretion of LH by the anterior pituitary gland to provoke testosterone biosynthesis and secretion by Leydig cells in the testis during the later fetal, immediate postnatal and pubertal stages of development. By contrast to the fetal testis, the fetal ovary produces neither gonadal steroids (e.g. estradiol) nor MIS. In target cells, testosterone, or its more potent 5 $\alpha$ -reduced metabolite, dihydrotestosterone (DHT), bind to the androgen receptor and promote masculinization of the urogenital sinus and external genitalia in the fetus, as well as the pubertal expression of secondary sex characteristics and the initiation and maintenance of spermatogenesis.

### *Klinefelter syndrome*

Seminiferous tubular dysgenesis due to 47XXY aneuploidy in subjects with Klinefelter syndrome represents the most common cause of testicular failure, with a reported incidence of 1:500 males. Other sex chromosome aneuploidies, 48XXYY, 48XXXYY and 49XXXXYY, also occur but with much reduced frequencies and are also males. 47XXY males may present with a variety of subtle, age-related clinical signs including hypospadias, small phallus or cryptorchidism in infancy; language delay, learning disabilities or behavioral problems in adolescence; followed by delayed or incomplete pubertal development with eunuchoid body habitus, gynecomastia (breast enlargement) and small testes with infertility due to progressively decreasing numbers of spermatogonia. At puberty, gonadotropin (luteinizing hormone, LH and follicle stimulating hormone, FSH) levels increase but testosterone concentrations remain relatively suppressed in accordance with the degree of testicular failure. Gynecomastia arises from the decreased testosterone:estradiol ratio. Despite impairment of seminiferous tubular function, intratesticular sperm retrieval and intracytoplasmic sperm injection for fertilization of eggs has been used successfully for a few subjects.

### *Turner syndrome*

The diagnosis of Turner syndrome is based upon the characteristics of short stature, gonadal dysgenesis leading to premature ovarian failure and infertility, typical dysmorphic features (epicanthal folds, high arched palate, low nuchal hair line, webbed neck, shield-like chest, pigmented nevi and inverted nipples), and abnormalities in the kidneys and heart. The karyotype is complete or partial absence of one of the X chromosomes (45X), although frequently accompanied by cell line mosaicism (45X/46XX). The presence of dysgenic gonad(s) and Y chromosome material increases the probability for development of gonadoblastomas. Hormone replacement therapy is necessary to promote normal female sex characteristics and to prevent cardiovascular complications and osteoporosis in affected subjects. Oocyte donation is an option for assisted reproduction.

### *XX males*

Subjects with an apparent 46XX karyotype but male phenotype are the result of translocation of a portion of the Y chromosome containing the testis determining SRY gene most often to the X chromosome. Subjects may have undescended testes (15%) and hypospadias (10%) and usually have small testes that may be soft early in life but become firm with increasing age. Testicular histology reveals no spermatogonia, a decrease in the diameter of the seminiferous tubules and Leydig cell hyperplasia, similar to that in Klinefelter syndrome. By comparison, 46XX males are shorter than those

with 47XXY karyotype. Testosterone production is low whereas gonadotropin levels are elevated.

### *XY gonadal dysgenesis*

Gonadal dysgenesis may be of the “pure” or “mixed” form where the former refers to the presence of aplastic or “streak” gonads on both sides and the latter to a unilateral streak gonad on one side and testicular tissue, usually within a dysgenic testis on the other side. The pure form may occur in subjects with a 46XY karyotype, whereas the mixed form commonly involves chromosomal mosaicism (45X/46XY) but also occurs in 46XY subjects with variable degrees of functional testicular tissue in each of the gonads. The etiology may be deletion of the Y chromosome or deletion/mutation of the SRY gene, or other genes associated with testicular development such as SOX9, SF1, or WT1.

## **Genetic lesions in the hypothalamic-pituitary-gonadal axis**

### *GnRH synthesis and release*

Gonadotropin releasing hormone (GnRH) neurons originate in the olfactory placode and migrate with olfactory neurons to the olfactory bulb and finally to the fetal hypothalamus, a position that enables their secretory product, GnRH, to access the hypophyseal portal circulation and reach the anterior pituitary. If this migratory process is disturbed, the outcome is hypogonadotropic hypogonadism (combination of low gonadotropin and gonadal steroid levels) with anosmia (lack of smell), referred to as Kallmann syndrome (KS), with a frequency of 1:8000 in boys. This syndrome is genetically heterogeneous with X-linked, autosomal dominant and autosomal recessive forms. The autosomal forms comprise 85% of the cases. Two genes can cause this condition: KAL1, encoding anosmin-1, is mutated in some X-linked forms of KS whereas KAL2, encoding the fibroblast growth factor receptor 1 (FGFR1), is mutated in some autosomal dominant cases. Other candidate genes implicated in KS are chromodomain helicase DNA binding protein 7 (CHD7) and G-protein coupled receptor prokinectin receptor-2 (PROKR2). Adult men with KS do not develop male secondary sex characteristics in the absence of androgen replacement and remain azoospermic unless treated with GnRH or gonadotropins; androgen therapy alone does not initiate spermatogenesis in these men.

A breakthrough in understanding the regulation of GnRH synthesis and secretion was the recent discovery that GPR54, a G-protein coupled receptor and its ligand, kisspeptin, trigger puberty in humans and experimental animals (Chapter 18). Kisspeptin is expressed in the arcuate nucleus and GPR54 in GnRH neurons. Subjects with inactivating mutations in GPR54

present with hypogonadotropic hypogonadism due to absence of GnRH synthesis and release. These subjects lack pubertal development but respond to exogenous administration of GnRH. In rare cases, congenital deficiency of leptin or its receptor can lead to severe early onset obesity and hypogonadotropic hypogonadism due to GnRH deficiency. Leptin regulation of gonadotropin secretion is likely due to its role in the release of neurotransmitters, such as neuropeptide Y, that affects GnRH secretion.

Although the GnRH gene is a natural candidate for mutations leading to hypogonadotropic hypogonadism, such mutations have not been detected. By contrast, inactivating mutations of its receptor, GnRHR, have been reported. Mutations of the GnRHR gene in males cause delayed puberty, decreased libido, poor masculinization and reduced testis size with asthenoteratozoospermia. GnRH mutations cause reduced ligand binding and/or reduced second messenger (inositol triphosphate) response to GnRH stimulation. In affected subjects, the gonadotropin levels are low with normal frequency of low amplitude peaks and responses to GnRH stimulation are variable.

#### *Gonadotropins and their receptors*

Mutations in gonadotropin subunits would be expected to directly impair fertility and reproduction, and thus would likely be eliminated from the gene pool. Hence, only a few sporadic cases of such mutations have been described. In addition to inactivating mutations, polymorphisms in these genes are suggested to be causative or contributing factors to mild disturbances of male reproductive function. The common  $\alpha$ -subunit is shared by the three gonadotrophins, luteinizing hormone (LH), human chorionic gonadotrophin (hCG) and follicle stimulating hormone (FSH), as well as by thyroid-stimulating hormone (TSH). Therefore, inactivating mutations of this gene would have significant consequences with respect to male hypogonadism, hypothyroidism and pseudohermaphroditism. In females, pregnancy would be seriously compromised by the absence of hCG during gestation. Consequently, no mutations in the  $\alpha$ -subunit gene have been detected in humans. Rare inactivating mutations of the LH $\beta$  subunit gene have been reported in men. These men are masculinized at birth presumably due to normal activity of hCG during pregnancy, but lack sexual maturation at puberty in the absence of pituitary LH and postnatal stimulation of Leydig cell testosterone biosynthesis.

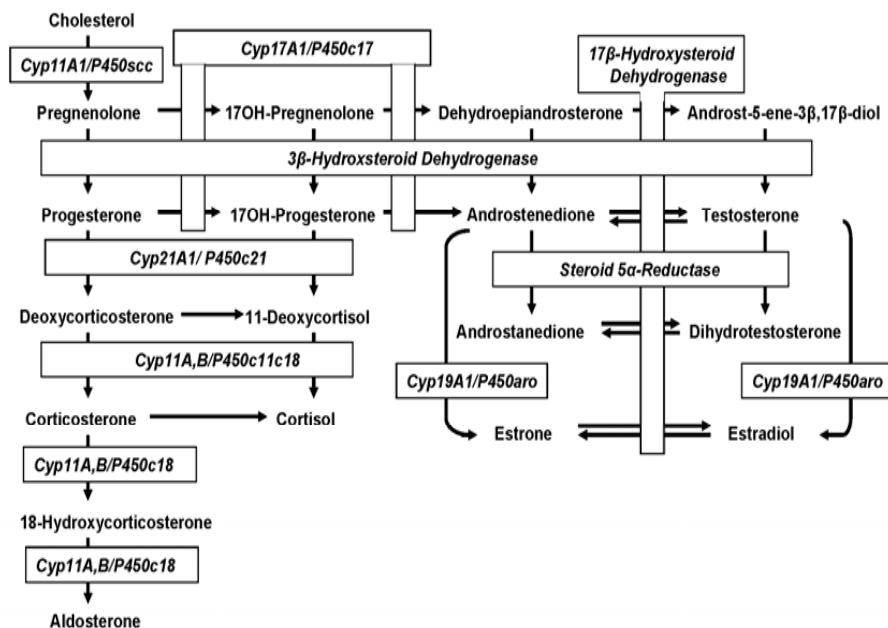
LH receptor (LHR) gene mutations occur in humans; a phenotype is observed when both alleles are inactivated due to homozygous or compound heterozygous mutations. The inactivating mutations may be complete or partial. With mutations of LHR, the testis is devoid of trophic stimulus and the Leydig cells fail to develop resulting in a condition referred to as Leydig cell

hypoplasia or agenesis. Testes have Sertoli cells and occasional immature germ cells. Subjects fail to masculinize due to the absence of fetal testicular androgens as well as impaired postnatal androgen biosynthesis in response to hCG/LH. In severe cases of Leydig cell hypoplasia, subjects have female external genitalia, low testosterone and high LH levels, normal FSH concentrations and absence of secondary sex characteristics, but do not have breast development at puberty. The low testosterone levels and absence of breast development distinguish these subjects from those with complete androgen insensitivity (as discussed below). In cases of partial LHR inactivation, testicular androgen production is sufficient to stimulate partial masculinization of the external genitalia represented by cryptorchidism, small phallus and/or hypospadias, thus resembling partial forms of androgen insensitivity syndrome. Gain of function mutations associated with constitutive activation of LHR have also been described in subjects with early onset familial male-limited precocious puberty, also termed testotoxicosis due to the chronologically abnormal elevation of testosterone production. Early onset of puberty causes short stature in adult age and the residual psychological impact of inappropriately early pubertal development, whereas testicular function and fertility are otherwise normal.

Interestingly, the FSH receptor (FSHR) and LHR genes are both located on chromosome 2p. Loss of function mutations in FSHR cause phenotypes in homozygous and compound heterozygous forms. Fewer FSHR than LHR mutations are known probably because the phenotypes associated with the former are less clinically striking. Although FSH action was presumed to be essential for Sertoli cell proliferation and function and for regulation of spermatogenesis, FSHR mutations in men have been reported to reduce testicular volume and lower sperm counts, but do not cause azoospermia. These findings suggest that normal androgen production in these men suffices to initiate and maintain spermatogenesis at a qualitatively and quantitatively reduced level that obviates a requirement for FSH bioactivity.

#### *Disorders of androgen biosynthesis*

Androgens and estrogens are made from cholesterol by a series of well-defined enzymatic steps (Chapter 5) (Fig. 2). Defects in these enzymes affect androgen biosynthesis by Leydig cells and virilization of the male fetus but do not interfere with regression of the Mullerian ducts. The enzymatic defects may be of variable severity, partial or complete, and may present in the newborn period as degrees of sexual ambiguity ranging from complete feminization to mild hypospadias or they may only become apparent at puberty. Severe defects in these enzymes that affect mineralocorticoid and glucocorticoid biosynthesis may present life-threatening circumstances in the newborn.



The first step in the steroidogenic pathway involves the entry of cholesterol into the mitochondria with the assistance of the steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO; also known as peripheral benzodiazepine receptor, PBR). Mutations in StAR cause congenital lipoid adrenal hyperplasia, a potentially lethal disease associated with severe deficiency of glucocorticoid and mineralocorticoid, as well as other steroids. Within the mitochondria, cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme, cytochrome P450<sub>scc</sub> (also known as CYP11A). Defects in this enzyme are very rare, likely due to embryonic lethality. In humans, pregnenolone undergoes 17 $\alpha$ -hydroxylation by microsomal P450<sub>c17</sub> (CYP17) primarily to 17-hydroxypregnenolone. 17 $\alpha$ -hydroxylase deficiency is extremely rare but

presents as a combination of female sexual infantilism and hypertension associated with gonadal and adrenal deficits, respectively, in steroid synthesis. 17-hydroxypregnenolone is then converted to DHEA by the dual 17,20-lyase activity of the same P450c17 enzyme. The ratio of 17,20-lyase to 17 $\alpha$ -hydroxylase activity of P450c17 determines the ratio of C21 to C19 steroids produced in the gonads and adrenals. This ratio is regulated post-transcriptionally by at least three factors: the abundance of the electron-donating protein P450 oxidoreductase (POR), the presence of cytochrome b5 and the serine phosphorylation of P450c17. Mutations of POR are a recently described disorder manifesting as the Antley-Bixler skeletal dysplasia syndrome, and a form of polycystic ovary syndrome. Androstenedione is produced by 3 $\beta$ -hydroxysteroid dehydrogenase activity primarily from DHEA, with only minimal quantities being derived from 17-hydroxyprogesterone in humans. Finally, 17 $\beta$ -hydroxysteroid dehydrogenase activity converts androstenedione to testosterone. This enzyme is present in the gonad but not the adrenal and its deficiency may cause genital ambiguity at birth but virilization at puberty.

### Defects in androgen action

*Steroid 5 $\alpha$ -reductase*

Abnormalities of androgen bioactivity can be due to inadequate conversion of testosterone to its more potent metabolite, 5 $\alpha$ -dihydrotestosterone (DHT). The effects of DHT are required within the genital tubercle and labioscrotal folds of the developing male fetus between weeks 7-12 of gestation to fully masculinize the external genitalia. Testicular testosterone and MIS production is normal so that Mullerian regression occurs and internal Wolffian duct structures develop to varying degrees. However, the sperm carrying ducts end blindly so sperm are necessarily absent in the ejaculate and prostate development is impaired. The genetic mutation is in the 5 $\alpha$ -reductase type 2 isoenzyme gene. Inheritance of 5 $\alpha$ -reductase deficiency is autosomal recessive and is most commonly found among isolated ethnic groups due to consanguinity. If naive subjects reach puberty, the external genitalia become further virilized with obvious phallic growth and development of a muscular body habitus and male body hair pattern. The hormonal profile is characterized by normal or elevated testosterone but low DHT levels relative to testosterone and higher than normal 5 $\beta$ - to 5 $\alpha$ -reduced urinary steroid metabolites.

### Androgen receptor

Complete or partial defects in response to testosterone and/or DHT are defined as androgen insensitivity (AIS); it is due to mutations in the X-linked

androgen receptor gene that prevent normal expression of androgen-responsive genes. In complete AIS, the phenotype of the external genitalia is female despite normal to elevated levels of testosterone and DHT. In the infant, inguinal or labial testes may be palpable, although they are most often discovered during exploration for an apparent inguinal hernia. The vagina is short due regression of the Mullerian ducts following secretion of MIS by testicular Sertoli cells. High testosterone levels result from elevated gonadotropins at puberty due to the lack of negative feedback at the hypothalamic-pituitary axis. However, peripheral aromatization of testosterone leads to high levels of estradiol that promotes breast development when unopposed by androgen action. Axillary and pubic hair is scant. If the diagnosis is not made in the infant, the clinical presentation may be primary amenorrhea and infertility at the expected time of puberty. The increased incidence of testicular tumors in the undescended testes during the third decade of life recommends orchiectomy followed by estrogen therapy. By comparison, partial AIS presents as highly variable degrees of genital virilization ranging from slightly virilized female genitalia to hypospadias, undescended testes and adolescent gynecomastia to micropenis or even isolated infertility. These subjects may present with ambiguous genitalia during the neonatal period with a hormonal profile of elevated testosterone and LH, characteristic of androgen insensitivity. Further diagnostic testing with androgen stimulation but inadequate penile growth in response to androgen is indicative of androgen insensitivity. Marked ambiguity and biochemical evidence of demonstrable androgen insensitivity may dictate a female sex of rearing as the partial defect may allow some masculinization in response to the pubertal rise in testosterone that is accompanied by gynecomastia. Prepubertal gonadectomy will prevent the latter circumstances. Recently, abnormal expansion of the trinucleotide repeat region encoding the polyglutamine sequence in the aminoterminal of the androgen receptor has been determined as the cause of spinal bulbar muscular atrophy in young men who may initially present with apparent hypogonadism. Additional studies have suggested that polymorphism of the glutamine repeat region may also influence male fertility.

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## Is there a trigger for puberty in the male? Should early or delayed puberty be treated? If so, how?

T.M. Plant and S.F. Witchel

Puberty in boys normally occurs between 10 and 17 years of age, when the individual matures sexually and becomes capable of producing sperm and reproducing. The physical manifestations of puberty in males include growth in the sizes of the testes and the phallus, development of pubic, axillary and facial hair, and adult apocrine odor, accelerated body growth, increased muscle strength, and changes in mood and behavior. Transition from boyhood to adolescence occurs because of two physiological processes, that typically are sequentially and independently activated. The first is adrenarche, which is a maturation of the adrenal cortex associated with increased secretion of dehydroepiandrosterone sulfate and other adrenal androgens. Adrenarche, the cause of which is poorly understood, leads to pubic and axillary hair, apocrine odor, and acne (pubarche). The second is gonadarche, which is comprised of increased secretion of testicular testosterone from the Leydig cells and initiation of spermatogenesis. All phenotypic aspects of pubertal development including spermatogenesis are the result of increased testosterone secretion. The stimulus for testosterone release is increased luteinizing hormone (LH) secretion from the pituitary gland, which in turn is driven by activation of a pulsatile (1 pulse every 2-3 hours) discharge of gonadotropin releasing hormone (GnRH) from axon terminals in the median eminence of the hypothalamus into a portal blood system supplying the pituitary. GnRH is synthesized by approximately a thousand neurons that are diffusely distributed throughout the hypothalamus. Pulsatile GnRH release also increases the secretion of follicle stimulating hormone (FSH), which acts in concert with testosterone at the level of testicular Sertoli cells to amplify the action of the androgen to maintain spermatogenesis at maximal levels. For puberty to be completed and for testosterone and spermatogenesis to be maintained in adulthood a sustained pulsatile GnRH stimulation of the pituitary is required.

The hypothalamic neuronal network responsible for pulsatile GnRH release develops in the fetus where it promotes external genital development, and remains functional during the first 6 months of postnatal life. This is reflected by elevated gonadotropin secretion and testicular testosterone release during infancy. The seminiferous tubule of the infant, however, is unable to respond to the “adult” hormonal milieu because androgen and FSH receptor signal transduction pathways in the Sertoli cell

have yet to mature. Later in infancy, intermittent hypothalamic GnRH release is greatly diminished leading to low gonadotropin levels that guarantee continued quiescence of the prepubertal testis. During childhood, the pituitary and testis are able to respond to stimulation with GnRH and gonadotropin, respectively, and therefore these components of the reproductive axis are not limiting to the onset of puberty.

The up-down-up pattern of GnRH release during the infant-childhood/juvenile-adolescent transitions indicates that there are two critical postnatal “switches” or “triggers” related to the onset of puberty: the first, operational in the infant, leads to suppression of GnRH release during the greater part of prepubertal development, and the second is responsible for reactivation of intermittent GnRH release at the termination of juvenile development.

The reduction in GnRH release during childhood and juvenile development is viewed to result from imposition of a neurobiological brake on the GnRH pulse generator during these developmental phases. A major component of this brake is accounted for by a reduction in stimulatory kisspeptin input to the GnRH neurons. Kisspeptin is encoded by the gene, *KISS1*, which is expressed in hypothalamic neurons located in the infundibular nucleus that surrounds the base of the third cerebroventricle. Kisspeptin, a potent stimulator of GnRH release, signals G protein coupled receptor 54 (GPR54 or KISS1R), which is expressed by GnRH neurons. *KISS1* expression is low during childhood and increases at puberty. Inactivating mutations of GPR54 are associated with absent or delayed puberty. Other neuropeptides, in particular neurokinin B, neurotransmitters (GABA and glutamate) and glial derived growth factors have also been implicated in this neurobiological brake.

Two control systems have been proposed to govern the timing of the neurobiological brake dictating the postnatal pattern of GnRH release. The first is a somatometer that posits that GnRH release during postnatal development is governed by a hypothalamic system that tracks circulating signals and reflects somatic growth. Thus, attainment of adult stature is coordinated with the attainment of fertility. The second posits a pubertal “clock” also resident in the hypothalamus and comprised of a hierarchical network of genes encoding for transcriptional factors.

Disorders of puberty may lead to delayed or early (precocious) puberty. Causes of delayed or absent puberty include primary deficiencies in secretion or action of GnRH and/or, gonadotropins, and testicular failure due to Klinefelter syndrome (47,XXY karyotype), single gene disorders, or gonadal dysgenesis (Chapter 17). Chronic illnesses and impaired nutrition may lead to reduced gonadotropin secretion. Laboratory evaluation for delayed puberty includes serum LH, FSH, and testosterone measurements

and a bone age X-ray to assess skeletal maturation. Delayed puberty due to testicular failure can be readily distinguished from hypothalamic and pituitary deficiencies by history, physical examination, and laboratory studies. While testosterone concentrations are subnormal in both cases, gonadotropin levels are elevated only in testicular failure. Chronic illnesses and impaired nutrition (including restricting calories to limit weight gain) can generally be diagnosed based on history, physical examination, and laboratory data. Specific treatments and improvement in caloric intake can thus be implemented accordingly to reverse the hypogonadism. Hormonal therapy to elevate testosterone levels using exogenous human chorionic gonadotropin, LH or testosterone may be necessary.

Constitutional delay of growth and development (CD) is a familial variant of normal in which boys develop signs of puberty later than their peers. Often, boys with CD are referred to the pediatric endocrinologist as they become aware of discrepancies between themselves and their peers in height, muscular development, athletic capabilities, and sexual development. For some boys, a delay in pubertal development may be associated with depression, anxiety, and school failure that may lead to major psychosocial dysfunction; short term hormonal therapy can alleviate some of the physical differences and lessen the impact of the psychosocial issues.

Early or precocious puberty occurs less commonly in boys. Potential consequences include tall stature during childhood, advanced skeletal maturation, and premature physical manifestations of puberty. The advanced skeletal maturation often leads to short stature in adulthood due to early epiphyseal fusion in the long bones. Precocious puberty may result from premature reactivation of pulsatile GnRH secretion caused by one of several factors including brain tumors and dysfunction of the hypothalamus. It can also occur as a consequence of central inflammatory disorders, post-radiation therapy, or brain trauma. GnRH receptor “super-agonists” such as leuprolide acetate and histrelin are used to treat these central causes of precocious puberty.

Precocious puberty may also result from excessive and premature GnRH independent androgen secretion caused by either congenital virilizing adrenal hyperplasias, familial autosomal dominant male limited precocious puberty (testotoxicosis), or Leydig cell tumors. Congenital adrenal hyperplasia represents a group of disorders featuring inactivating mutations in steroidogenic enzyme genes that result in low levels of cortisol that in turn lead to increased pituitary secretion of adrenocorticotrophic hormone (ACTH) and adrenal androgen secretion. Testotoxicosis is due to activating mutations of the LH receptor gene. Tumors located in the liver, mediastinum, or central nervous system may secrete human chorionic gonadotropin that binds to testicular LH receptors resulting in increased testosterone secretion and

precocious puberty. Therapy is directed at the underlying disorder. In some instances, these peripheral causes of precocious puberty are complicated by secondary development of increased pulsatile GnRH secretion.

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## Clinical male infertility: Epidemiology and basic evaluation

P. Chan

It is estimated that one in seven couples have problems conceiving. The incidence of infertility is similar in most countries regardless of their level of development. Over 80% of couples who have “regular” frequency of sexual intercourse (every 2 to 3 days) and who do not use any means of contraception will achieve pregnancies within one year. Approximately 92% of couples can achieve pregnancy within 2 years. Although in many cultures and societies the cause of infertility is assumed to be on the female side, in reality, roughly a third of infertile couples are due to problems with the man, another third due to problems with the woman and another third due to a combination of both male and female factors. Thus, evaluation of male infertility is essential in counseling couples for their fertility options.

Clinical evaluation of male fertility begins with a detailed history and physical examination, which generally will provide valuable information to guide what additional laboratory investigations or imaging studies to complete the evaluation. The two main purposes of male fertility evaluation are: i) to identify any modifiable factors that can improve the fertility status of the man; and ii) to identify any underlying serious conditions, such as testis cancer, osteoporosis, endocrinological and genetic problems that present first as infertility.

Important information to be obtained from the patient's history includes the duration of infertility, previous history of natural fecundity, or fertility treatment. Important past medical history such as past or current sexually transmitted infection, genital urinary problems, malignancy, congenital, developmental or genetic problems, may point to the cause of infertility. Surgical history such as previous hernia repair, scrotal surgery such as orchiopexy for undescended testes, history of trauma to the pelvis or genital-urinary tract may be associated with an increased risk of infertility. A list of medication used currently or in the past should be obtained. Family history of infertility, endocrinological disorders, malignancy and other genetic conditions such as cystic fibrosis may also suggest the underlying cause of infertility. A detailed sexual history and social history including the use of tobacco and recreational drugs, anabolic steroids or exposure to any other gonadotoxins is also required as part of the evaluation.

A thorough physical examination begins with an evaluation on the general physique of the man, focusing on the proper development of secondary sexual characteristics. Additional signs associated with infertility include gynecomastia (which may indicate endocrinological or hepatic

problems), anosmia (indicating hypogonadotropic hypogonadism) and situs inversus (associated with sperm defects). Genital examination should be performed in a warmed room and should include the evaluation of the phallus for any defects such as hypospadias (downward-shift of urethral opening), anomalies in the development of the scrotal wall and the contents. Testicular size and texture and the bilateral symmetry are important. Testis cancer, which is known to be associated with infertility, may present as a painless hard mass. Bilateral soft and small testes may be associated with hypogonadism. An orchidometer may be helpful to objectively determine the testicular volume. Men with Klinefelter's syndrome (Chapters 16, 17), one of the most commonly diagnosed genetic cause of infertility, may have hard testis with less than 3 cc in volume. Determination of the presence of vasa deferentia bilaterally, the position and fullness of the epididymis, the presence of inguinal hernia are all important part of the genital examination. Inspection and palpation for varicoceles (abnormal enlargement of veins in the scrotum that drain the testes) in the spermatic cord should be done when the patient is in an upright position. Finally, a digital rectal examination of the prostate may sometimes reveal i) tenderness that may suggest inflammatory or infectious process of the genital tract; ii) a mass which may represent cyst, stone or prostate cancer.

The basic laboratory evaluation includes two semen analyses (at least 1 to 2 months apart) and morning serum hormonal profile. In the absence of any significant findings in the history and physical examination, some investigators believe that if a single semen analysis demonstrated sperm count above 60 million/ml (four times the WHO reference values) with no additional parameters below the WHO reference values (Chapter 12), no additional evaluation is required. It must be emphasized that without a detailed history and a thorough physical examination, semen analysis alone, even if all parameters met the WHO reference values, does not rule out the presence of correctable male-factor infertility. Although semen analysis is the most commonly used laboratory evaluation for male fertility, other than at the extreme ends (extremely low or high parameters), semen analysis results have a weak predictive value on the probability of success of achieving pregnancy with the female partners naturally or with assisted reproductive technologies.

Basic blood tests for male fertility evaluation include a morning total testosterone and follicle-stimulating hormone (FSH). Evaluation of estradiol level, which has a role to negatively suppress the level of gonadotropins, can also be useful. In the presence of hypogonadism (low testosterone level), the levels of luteinizing hormone (LH) and prolactin should be determined. Interpretation of serum hormonal profile should be done in conjunction with the clinical history and physical examination. Men with azoospermia or

severe oligospermia with an FSH level above 10 IU/L is indicative of non-obstructive azoospermia or testicular dysfunction. If the level of FSH is in the low normal range, it may indicate the presence of obstruction in the excurrent ductal system leading to the low level or absence of sperm in semen. FSH levels well below normal (< 1.5 IU/L) are consistent with hypogonadotropic hypogonadism. Determination of inhibin-B level can also provide important information on the fertility status. However, the assay for inhibin-B is costly and is not widely available in most laboratories.

In men with azoospermia or severe oligospermia, genetic evaluation may be indicated. Currently, in azoospermic men with absence of the vas deferens, he and his partner should be evaluated for mutation in the cystic fibrosis transmembrane conductance regular (CFTR) gene. In men with azoospermia or severe oligospermia due to testicular dysfunction, blood test for karyotype and Y-chromosome microdeletion should be done to evaluate chromosomal anomalies. Many of these men with azoospermia or severe oligospermia may still be candidates for advanced assisted reproductive technologies to procreate. Since there are risks that they may pass similar or related genetic problems to their offspring, proper genetic counseling is important for couples who have genetic causes of infertility.

Additional evaluation for male fertility includes imaging studies such as i) trans rectal ultrasound for evaluation of prostatic cysts and ejaculatory duct obstruction; ii) scrotal ultrasound for evaluation of epididymal cysts, testis mass, varicoceles and other scrotal pathology; iii) magnetic resonance imaging for evaluation of pituitary prolactinoma that may lead to hypogonadism. Diagnostic testicular biopsy may occasionally be used to confirm the presence of normal spermatogenesis in men who are azoospermic or severely oligospermic in preparation for subsequent microsurgical reconstruction of the male excurrent ductal system to bypass the obstruction.

The use of various molecular assays to determine sperm chromatin integrity has recently generated a lot of interest among clinicians (Chapter 13). Common techniques such as the comet assay, TUNEL assay, sperm chromatin structure assay (SCSA®) have been applied in human sperm by various investigators. Men with clinical infertility as a group tend to have poorer sperm chromatin integrity than men who have no difficulty achieving natural fecundity. Some studies have demonstrated a correlation on the sperm chromatin integrity with reproductive success while others fail to demonstrate a significant predictive value of these assays on reproductive outcomes. Currently, according to the guidelines published by the American Society of Reproductive Medicine, sperm chromatin integrity assays is not cost effective to be used routinely for all couples being evaluated for infertility. Further studies will be required to fully explore the clinical values of

these assays in predicting success in fertility and the risks of adverse reproductive outcomes.

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## Why evaluate the infertile male in the era of ART?

### *Medical and surgical therapies for male infertility*

M. Goldstein

With in-vitro fertilization (IVF) employing intracytoplasmic sperm injection (ICSI), live births can be achieved with only a handful of sperm. So why bother evaluating the male?

- 1) There is a 9 to 20 times higher incidence of testicular cancer in infertile men compared to age-matched controls.
- 2) There is a 30 to 100 times higher incidence of genetic abnormalities in infertile men.
- 3) Varicocele, a dilation of the pampiniform plexus of veins, affects both spermatogenesis and Leydig cell function. At every age, men with varicocele have lower testosterone levels than men without varicoceles.
- 4) Most couples prefer to conceive as naturally as possible. Treatment of the male often allows upgrading of fertility status: from adoption or the use of donor sperm, to testicular or epididymal sperm retrieval for IVF/ICSI. Treatment can induce appearance of sperm in the ejaculate, allowing IVF/ICSI without the need of surgery for sperm retrieval. Men with only enough sperm for IVF/ICSI can be upgraded to intrauterine insemination (IUI).
- 5) Finally, treatment of the male may allow natural conception.

### **Obstructive azoospermia**

All azoospermia is either obstructive, wherein the male has normal production, or non-obstructive, where the problem is lack of sperm production. In obstructed men, presence of circulating antibodies against sperm confirms the presence of spermatogenesis and obviates the need for testis biopsy. The most common causes of obstructive azoospermia are: prior vasectomy, iatrogenic injury due to prior hernia repair (vasal obstruction), hydrocelectomy (epididymal obstruction), orchiopexy for torsion (obstruction from a stitch going through the epididymis), or any other scrotal or inguinal procedure. Vasovasostomy is a highly successful microsurgical procedure for reversing obstruction. Microsurgical vasovasostomy (Fig. 1) results in the appearance of sperm in the ejaculate in over 90% of men and pregnancy rates, that are dependant on female partner age, vary from 50 – 80%, depending on time of obstruction. Epididymal obstruction requires

vaso-epididymostomy, with current techniques yielding patency rates of over 80% and pregnancy rates of 40%. Men with congenital absence of the vas deferens (usually associated with CFTR mutations) or with unreconstructable obstructions, are successfully treated with microsurgical epididymal sperm aspiration and IVF/ICSI.

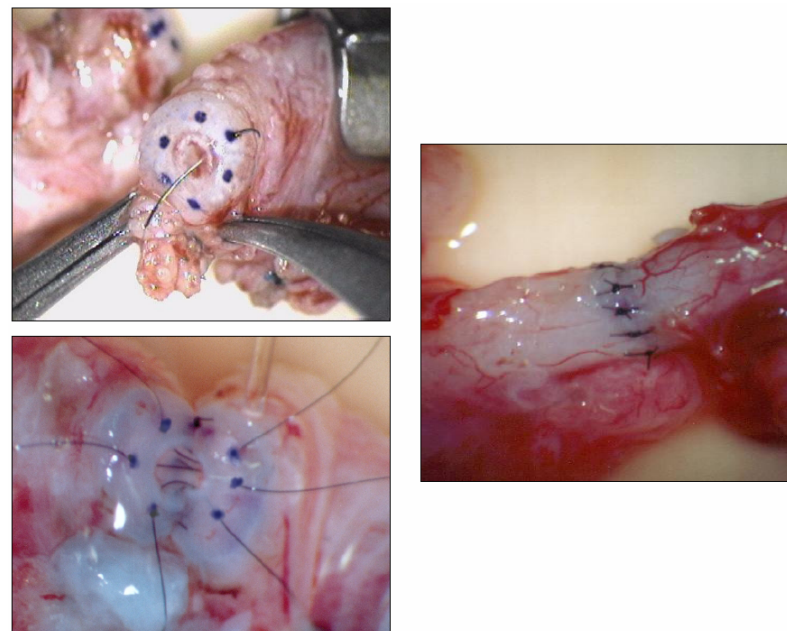


FIG.1. Surgical procedures involved in microsurgical vasovasostomy.

Retrograde ejaculation (often the first presenting sign of diabetes mellitus) can be treated with sympathomimetic drugs or retrieval of sperm from the urine for use with either IUI or IVF/ICSI. Anejaculation, due to either spinal cord injury, diabetes mellitus, retro-peritoneal lymph node dissection for testicular cancer, or psychogenic anejaculation, should be tried first with vibratory stimulation. If that is not successful, electroejaculation should be used; it is highly successful in obtaining sperm.

### **Non-obstructive azoospermia**

Endocrinopathies such as hypogonadotropic hypogonadism (H-H), called Kallmann's Syndrome if anosmia is present (Chapter 18), usually present as failure to go through puberty due to lack of testosterone and is caused by

a lack of hypothalamic GnRH (LHRH). Therefore, the pituitary gonadotropes are not stimulated to produce LH and FSH. Treatment entails replacement of pituitary hormones with LH, and, once normal testosterone levels are achieved, FSH is added. After six to twenty four months of treatment, the majority of such men goes through normal puberty and begins to have the appearance of sperm in the ejaculate, with good pregnancy rates.

Klinefelter's Syndrome (47XXY) may present in a similar fashion, with the failure to go through puberty, and feminized habitus (Chapter 17). Unlike H-H patients, who have undetectable levels of testosterone, LH and FSH, Klinefelter's men have low testosterone levels, but elevated LH and FSH (i.e. hypergonadotropic hypogonadism). Testosterone replacement will get them through puberty, but this will not induce spermatogenesis. These men are treatable with testicular sperm extraction, which is successful in obtaining sperm in 60% of them.

Men with micro deletions of the Y chromosome in the regions AZFa, AZFb and AZFc usually present with non-obstructive azoospermia. Men with an AZFc deletion are most likely to have either rare sperm in the ejaculate or 60% of the time on testicular microdissection, adequate sperm can be found for use with IVF/ICSI. Men with complete deletions of the AZFa or b segment have never had sperm found.

Idiopathic non-obstructive azoospermia is challenging to treat. If men have palpable varicoceles, microsurgical repair will induce the appearance of sperm in the ejaculate in about 50% of the time. Empiric treatment of men with low serum testosterone levels, using either clomiphene citrate or the aromatase inhibitors Arimidex or Teslac, has resulted in sperm in the ejaculate in some of these men. When all treatments have failed, these men are candidates for testicular microdissection for sperm retrieval for ICSI with a success rate of 60% and live delivery rates of 40% when sperm are successfully retrieved.

### **Oligoasthenoteratozoospermia**

Isolated defects of sperm count, motility or morphology are rare. The majority of infertile men present with low counts, impaired motility and abnormal morphology. The most common etiology for this is varicocele, found in 30% - 40% of infertile men, 70–80% of men with secondary infertility, but only 15% of the general population. Of all men who have varicoceles, approximately 20% are grade III or large varicoceles. These are visible through the scrotal skin, and are the ones most likely to respond to treatment. This means that approximately 3% of all men in the general population have grade III varicoceles. Prophylactic varicocelectomy in young men or boys with grade III varicoceles can prevent future infertility and

androgen deficiency. Microsurgical techniques for the repair of varicocele make it extremely safe and effective. There is a significant increase in sperm count and quality in 70% of men undergoing the procedure with pregnancy rates of over 40%. In addition, microsurgical varicocelectomy significantly increases testosterone levels and may be, in the future, accepted as a treatment for androgen deficiency.

When there is no identifiable etiology, empiric treatments with clomiphene citrate, which combines with estrogen receptors and therefore blocks the negative feedback to the hypothalamus and pituitary and increases LH and FSH levels, has been reported to improve sperm production in some men.

### **Prevention**

The testis manufactures approximately 50,000 sperm per minute and is regarded the canary of the human body. It is exquisitely sensitive to the adverse effects of environmental disrupters and gonadotoxins (Chapters 28-30). Lifestyle recommendations include avoiding gonadotoxins such as alcohol, marijuana and cocaine. Be aware of the adverse effect of prescribed medications, such as the sulfa drugs used for treating inflammatory bowel disease, calcium channel blockers used for treating hypertension, as well as chemotherapeutic agents and radiation therapy used for treating cancer. All post-pubertal boys and men in whom chemotherapy or radiation therapy is planned should be counseled on the importance of sperm cryopreservation prior to treatment (Chapter 14). Experimentally, prepubertal boys with cancer who will undergo chemotherapy or radiation therapy may have testis tissue removed and cryopreserved for possible future maturation in vitro.

### **Summary**

In summary, there are many compelling reasons for treating male infertility. Evaluation and treatment by a specialist trained in male infertility and microsurgery results in optimal outcomes. Collaboration with specialists in assisted reproductive technology optimizes outcomes for infertile couples allowing the majority of men to have their own children.

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## What are the assisted reproductive technologies for male infertility?

### *Indications for IVF/ICSI/IUI, Surgical sperm retrieval techniques*

P.N. Schlegel

Male fertility problems can occur when sperm are limited in number or function. In some cases, sperm washing is used to concentrate spermatozoa and deliver them through the uterus at the time of ovulation. In order for this treatment (referred to as intrauterine insemination [IUI]) to be successful, typically 5 million motile sperm must be present in the ejaculate. A more advanced technique for enhancing the interaction of sperm and egg is in vitro fertilization (IVF). IVF is an involved process that includes treatment of the woman using a series of hormone injections to stimulate the ovaries to produce multiple eggs, egg retrieval, and fertilization of those eggs outside of the body. A limited number of the fertilized eggs (embryos) are then transferred back to the woman's uterus after incubating in the laboratory for several days. It was initially recognized that impaired sperm would not fertilize eggs very efficiently, even when sperm are put directly next to the eggs in the laboratory. Indeed, it was recognized early on in IVF that if sperm were abnormal, then IVF was not very successful. Conditions where very impaired sperm are present include men who have very few sperm in the ejaculate, when motility of sperm is severely impaired, or the shape of the sperm was very abnormal. All of these conditions may be present for men with severely impaired sperm production. When there is severe deficiency of sperm number and/or limited ability of the sperm to fertilize during IVF, then the adjunctive treatment of intracytoplasmic sperm injection (ICSI) may be required to provide any reasonable chance of pregnancy to occur for these patients.

The solution of ICSI to enhance the ability of impaired sperm to fertilize an egg was identified in 1991 by Drs. Gianpiero Palermo and Andre Van Steirteghem in Belgium. During an attempt to place a sperm close to an egg, Dr. Palermo accidentally injected a spermatozoon directly into an egg. The injected egg fertilized, developed into an embryo, was transferred back to the woman's uterus and resulted in a pregnancy with delivery of a normal, healthy child. With this "accident," a revolution in fertility treatment (especially the treatment of men with severe fertility problems) was started. Subsequently, ICSI has been used to expand the spectrum of male-factor infertility cases that can be treated for infertility. Whereas sperm from the testis or epididymis were thought to be incapable of fertilization, ICSI has changed our view of the fertilizing ability of "immature" sperm retrieved from the male reproductive tract.

Currently, not only are sperm of limited number (oligozoospermia), severely impaired motility (asthenozoospermia), and sperm that are not normally shaped (teratozoospermia) possible to use for ICSI, but sperm taken from the epididymis or testis that have not passed through the male reproductive system can also be used for ICSI. The development of ICSI encouraged us to search more extensively for sperm in the testes, not only for men who have reproductive tract obstruction, but also for men with sperm production so poor that no sperm are present in the ejaculate (azoospermia).

In order to perform the single-sperm injection technique (ICSI), in vitro fertilization is necessary. In vitro fertilization involves the stimulation of a woman's egg production in a controlled manner so that multiple eggs are produced in the given month or "cycle" of treatment, rather than the usual one egg per month. Initial treatment with medications to suppress a woman's natural control of ovulation is given, followed by a series of hormone injections over a 3-4 week period of FSH-like fertility drugs. The final ten days or so of hormonal stimulation requires close monitoring of the woman's response to these drugs with daily serial ultrasound and blood tests. When optimal development of the eggs is seen, based on the development of follicles (fluid containing structures on the ovaries) and increased female hormone levels, then a final priming injection of a natural hormone (hCG), mimicking the physiological "LH-surge" that occurs before ovulation, is given to stimulate final egg development. Eggs are retrieved using a needle, directed by ultrasound through the vagina into these follicles, under a light general ("twilight") anesthetic. Sperm may then be injected into the eggs (oocytes) outside of the body ("in vitro").

The fertilized oocytes (now embryos) are maintained in culture for 3-5 days before transfer back to the woman. The chance of an individual embryo implanting and developing into a fetus is limited to 20-50%, depending on how well the embryo developed prior to transfer. Overall, the chance of pregnancy for each IVF attempt varies based on the age of the female partner. The chance of delivering a child for an individual IVF attempt from initial stimulation of egg production averages about 30%, but the chance of pregnancy when a woman is over 40 years of age may drop to about 10%.

Since the sperm that are selected for IVF and ICSI would not have naturally fertilized eggs in the past, concern exists about the risk of potential birth defects after application of IVF/ICSI (Chapter 23). To-date, the risk of birth defects does not appear to be any higher with sperm selection during ICSI, although a small but statistically significant increase in chromosomal abnormalities occurs after ICSI. The increase of chromosomal abnormalities in children from 0.2 to 0.8% with ICSI may occur because of abnormalities in the man providing sperm or the ICSI procedure itself.

The development of the advanced reproductive techniques of IVF and ICSI have revolutionized the treatment of severe male infertility. Many men are now

routinely treated using IVF/ICSI who would not have naturally fathered children before. Many doctors believe that specific treatment of male abnormalities make these advanced reproductive treatments unnecessary, because natural pregnancy can occur if improved sperm production can be obtained. ICSI has allowed new groups of men to be treated, including men who require sperm retrieval because of a lack of sperm in the ejaculate. The outcomes of these treatments continue to be studied.

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## Are there genetic factors associated with male infertility?

D. Lamb

The genetic basis of male infertility probably represents one of the most important, yet under emphasized, causes of male infertility. The current diagnoses of male infertility are largely descriptive, i.e., cryptorchidism or failure of testicular descent, testicular failure, idiopathic infertility (the cause is unknown). These diagnoses reflect a relatively poor understanding of the processes regulating the development and function of the male genital tract, the process of spermatogenesis, ejaculation and fertilization. Each of these processes is complex with the expression of thousands of genes thought to be required and accordingly difficult to study. Yet the genetic causes of infertility should be an area of importance in reproductive medicine. Assisted reproductive technologies have been developed to overcome sterility allowing otherwise infertile couples to become biologic parents (Chapter 21). These technologies include in vitro fertilization (IVF, test tube babies), intracytoplasmic sperm injection (ICSI) used together with IVF and preimplantation genetic diagnosis to attempt to select embryos free of specific genetic diseases. Yet in depth thought about these techniques suggests that they are used to circumvent natural evolutionary pressures. In essence, an infertile male represents the end of that genetic line. In nature, infertility is a “genetic lethal” condition as the defective genes causing infertility (and perhaps genetic syndromes) cannot be passed on to future generations. Assisted reproductive technologies now bypass this evolutionary checkpoint.

What are the genetic problems currently known causing male infertility? If we consider the most simple examination of genetic information, inspection of the chromosomes by a karyotype analysis is the most superficial, but certainly a very important assessment. A karyotype is similar to looking at the volumes of an encyclopedia in the library. The number of volumes are checked and whether large portions are missing. With this approach, a number of genetic defects are recognized. Certainly chromosome abnormalities (both numerical and structural) account for a significant percentage of male infertility. With no additional diagnostic or physical evaluation, nearly 6% of infertile men will be found to have a chromosome defect. For example, Klinefelter syndrome (a chromosome defect with extra X chromosomes present—XXY, XXXY or XXXXY) accounts for about 14% of non-obstructive azoospermia (no sperm in the ejaculate due to a sperm production problem) (Chapter 17).

Klinefelter syndrome is an example of a numerical chromosome defect in

which a whole chromosome is gained or missing. More complex chromosome defects can be present with the individual having a mixture of cells or mosaicism (XY, XO, XYY, and so on). Structural chromosome defects in which part of a chromosome is missing, duplicated or misplaced (analogous to missing or duplicated chapters, chapters out of order or backwards) such as translocations, inversions, duplications, deletions can cause male infertility as well. For example, Y chromosome microdeletions, in which the missing portion is too small to be visualized on karyotype analysis but evident with more advanced molecular diagnostic tools (analogous to missing pages in a chapter) are present in about 8-12% of men with severe infertility such as non-obstructive azoospermia and a lower percentage of oligospermic men (low sperm count in the ejaculate).

At the level of individual genes, mutations or deletions can certainly be present as well. This is an area of active research investigation and much of our knowledge comes from studies in animal models. Disruption of genes encoding proteins involved in sex determination, sex development, steroid or protein hormone biosynthesis, metabolism or receptor action, genes involved in the paracrine (cell-cell) signaling in the testis by growth factors, cytokines and their receptors, genes involved in structural aspects of spermatogenesis and cell-cell interactions, the formation and function of the sperm and fertilization can cause male infertility. A challenge to investigators is the complexity of the signaling processes regulating these important steps required for fertility, making dissection of specific defects difficult in individual patients. However, as discussed below, there are several examples that provide important insights into the significance of defining these defects.

Men with obstruction of the male reproductive excurrent ductal system associated with congenital bilateral absence of the vas deferens (CBAVD) are now known to have mutations in the gene for the cystic fibrosis transmembrane regulatory protein or CFTR, which encodes an ion channel that pumps chloride out of cells. The most common mutations of the CFTR gene are those associated with cystic fibrosis. The observation that cystic fibrosis patients had co-existing CBAVD led investigator to ask if CBAVD patients who do not have cystic fibrosis had mutations in the same gene. The CFTR gene is huge with over 1300 different mutations identified to date. Not surprisingly, the mutations causing cystic fibrosis differ (“severe” mutations) from those causing CBAVD (“mild” mutations). Men with CBAVD may have mutation in only one allele or two mild mutations in each allele. Alternatively, a severe cystic fibrosis mutation may be found on one allele with a mild one on the other. 5T allele polymorphism in a non-coding region (intron 8) of the CFTR gene, commonly found in CBAVD men in association with a CFTR mutation in the other allele, can result in decreased amount of CFTR protein synthesized.



Assisted reproduction with surgical sperm retrieval for ICSI is practically the only hope for these men to father genetic children. For the CBAVD male it is assumed he has a mutation in the CFTR gene. The spouse is usually tested for the most common mutations causing cystic fibrosis (about 30-50 out of ~1300 mutations). Accordingly, even when the spouse was tested “negative” for CFTR mutation, unless the couple undergoes expensive evaluation of the entire sequence of the CFTR gene, they remain at risk (albeit lower if the most common mutations are not present in the female partner) of conceiving a child with cystic fibrosis or CBAVD. In the past, before this genetic cause of CBAVD was identified, couples underwent assisted reproduction with the risk of the birth of an affected child. The CBAVD-CFTR story represents an example of a technology (the assisted reproductive technologies) being developed before the clinical consequences of the method was understood.

Finally, because these assisted reproductive technologies overcome infertility, are they safe for the offspring? Generally, the children appear relatively healthy although there is an increased incidence in low birth weight and birth defects (particularly those affecting the genitourinary system) requiring surgical correction. There is a slight increase in the incidence of genetic imprinting disorders, such as Beckwith-Wiedemann. The majority of the children born seem healthy. Obviously, long-term studies of the safety and efficacy of these procedures are required.

For infertile couples, understanding the cause of their infertility is important. It allows them to make educated decisions regarding their choices to use assisted reproductive technologies, to use donor sperm, adopt or remain childless. This is particularly important when a genetic cause of infertility is known, as the defect can be transmitted to the offspring. In addition, because some genetic causes of infertility may also cause systemic abnormalities in the infertile male (or the offspring), in the future, advanced genetic testing to diagnose the cause of infertility will be of critical importance.

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## How can we assess the health outcomes in ART-conceived children?

### *Ethical issues in andrology*

**T.J. Walsh and M.S. Croughan**

### Introduction

The last four decades have seen a revolution in the treatment of infertility, beginning with the birth of Louise Brown, conceived by *in vitro* fertilization (IVF) in the United Kingdom in 1978 (Steptoe and Edwards, 1978). IVF overcame many boundaries to natural pregnancy by allowing an egg to be fertilized outside the body, with placement of resulting embryos into the mother's uterus. Although this technology bypassed certain impediments to natural pregnancy, would-be fathers were still required to have adequate sperm in the ejaculate that could be used for fertilization.

This paternal prerequisite was all but eliminated in 1992 by the advent of intracytoplasmic sperm injection (ICSI), whereby even men who produce very few sperm are able to foster pregnancy via the direct injection of a single sperm into the egg itself. This technology has been advanced further by the realization that men without sperm in the ejaculate can foster pregnancy via the direct attainment of sperm from the testis itself. Thus, by this technology, many aspects of natural selection are eliminated at the level of egg fertilization. Certain genetic causes of male infertility which normally would have been lethal for the germ-line, can now be passed to the next generation (Chapter 22). Further, even in cases where multiple sperm are available in the ejaculate, sperm are “manually” selected based upon their appearance rather than their natural ability to fertilize an egg.

Indeed, since the introduction of IVF with and without ICSI, over one million children had been born by 2005 worldwide with the use of this technology. Importantly, the use of ICSI has increased dramatically, even in the absence of severe male infertility. Unlike many other therapies in medicine, Assisted Reproductive Technologies (ARTs) did not undergo rigorous, multi-phased trials to determine outcomes and to identify adverse perinatal or childhood outcomes. As a result, couples who undergo treatments for infertility, especially those treated with ICSI, may do so without a complete understanding of short and long-term risks to their offspring.

### Barriers to the study of ART outcomes

Before attempting to interpret the available data on reproductive outcomes following ART, it is important to understand the many difficulties associated with this field of study. First are difficulties in the selection of an appropriate group of children to whom those conceived through technology can be compared. This speaks to the fundamental question: do adverse outcomes occur as a direct result of the cause of infertility (e.g., physiologic or genetic abnormalities), or as the result of the applied technology, or some combination of the two? To fully answer this question, studies must compare children conceived by infertile couples through ART not only to those spontaneously conceived by fertile couples, but also to those spontaneously conceived by infertile couples. Further, as ICSI is now applied to cases other than the most severe forms of male infertility, children conceived via ICSI by fathers with severe semen impairments must be compared to children conceived by the same technology to fathers with normal semen parameters. The assembly of such comparison populations is both difficult and costly.

A second issue in studying health outcomes is the confusion that is created by the many other factors that are associated with both infertility and with adverse perinatal and childhood outcomes, and that may or may not mediate the association between infertility and adverse outcomes. Examples include advancing maternal and paternal age, and multiple gestations. Thirdly, it is difficult to objectively assess the outcomes that occur in offspring conceived via ARTs. These difficulties include screening biases introduced by the increased scrutiny of ART-conceived children by parents and providers relative to those conceived naturally, the lack of long-term and consistent follow-up on both study subjects and comparison groups, and the lack of agreed upon standards by which adverse outcomes (including congenital anomalies) are classified. Lastly, skill in applying ART has rapidly and continually evolved over the last three decades, whereby the outcomes that are measured in children today are the result of technology from many years ago. This includes changes in embryo culture techniques, in the duration of embryo culture, and in the size and number of embryos transferred into the uterus. As a result, assessment of outcomes has proven to be a “moving target”.

### Health outcomes in ART offspring

In spite of the aforementioned difficulties, many studies have endeavored

to understand the potential associations between ART and adverse health outcomes in offspring. Overall, the use of ART appears to be safe; however, retrospective data suggests that infertility and/or its treatments may confer increased risk of certain adverse health events, both in the perinatal period and in childhood. For clarity, discussions of these health outcomes are provided in the following categories: 1) genetic disease; 2) perinatal; and 3) childhood.

#### *Genetic risk*

Many forms of male and female infertility have an underlying genetic etiology. Certain abnormalities that were at one time not transmissible to offspring due to insurmountable infertility, can now be bypassed with the use of technology, in particular, ICSI. Specific examples include cystic fibrosis, a severe pulmonary disease that in most men is accompanied by congenital bilateral absence of the vasa deferentia. As a result, this fatal mutation can now be directly transmitted to offspring. Deletions of certain genes on the Y chromosome can lead to male infertility, and when passed to a male offspring, can perpetuate and even worsen this “infertile phenotype”. Beyond these infertility phenotypes, offspring conceived by ART appear to have higher risk for genetic imprinting disorders and chromosomal aneuploidies compared to those conceived naturally. Recent evidence has suggested that some infertile men may have defects in their ability to repair DNA, and that such defects may confer a higher risk for certain cancers, and are transmissible to offspring.

#### *Perinatal risk*

Adverse health outcomes occurring in offspring in the perinatal period are virtually inseparable from those that occur to mother and fetus. Clearly, much of the risk of adverse events is resultant from multiple gestations. The use of ART has historically conferred an increased risk of multiples, as more than a single embryo is often transferred to the maternal uterus in an effort to increase the chance of pregnancy. The association between multiple gestations, preterm labor, low birth weight, and prolonged neonatal intensive care has been well described. Importantly, some data suggest that even when multiple gestations are accounted for, infants conceived by IVF are at higher risk for these adverse events, and those conceived with the use of ICSI have higher risk still.

#### *Childhood risk*

Despite the inconsistencies in defining and categorizing congenital malformations and in acquiring long-term follow-up data, several large studies have demonstrated a consistently higher occurrence of congenital

malformations in ART-conceived offspring relative to naturally conceived children. Of particular concern has been the increased occurrence of certain genitourinary abnormalities that require surgical correction and may confer long term health risk to adult males. Importantly, studies have compared outcomes in children conceived by infertile couples with the use of ICSI to children spontaneously conceived by infertile couples, and have found no difference in the rates of minor or major malformations, suggesting that increased risk may have its origin in the genetics of the couple, and not in the application of technology.

Whether or not children conceived through ART have an increased risk for neurodevelopmental abnormalities is not well established. This particular area of study has been hampered by limited and variable follow-up among offspring. However, recent data from a large cohort of children conceived by infertile couples with and without the use of ART, suggests that they may have higher risk of such disorders compared to children conceived by fertile couples. In this study, children conceived by infertile couples were three times more likely to have been diagnosed with cerebral palsy, mental retardation, autism, seizure disorder, or cancer when these conditions were considered together as “severe” outcomes. Children conceived to infertile couples also were 40% more likely to be diagnosed with a “moderate” health condition such as attention deficit hyperactivity disorder, attention deficit disorder, a learning disability, behavior disorder, developmental delay, a serious vision disorder, or a serious hearing disorder by six years of age when compared to children conceived by fertile couples. However, the authors pointed out that these conditions were still very rare in these children and that the majority of children conceived to infertile couples were healthy and did not have significant health issues. They also reported that there does not appear to be significant differences in the health outcomes of these children based on the infertility treatments used to conceive the children. Rather, it appears that factors that initially contributed to the infertility may continue to cause problems during pregnancy and in the resulting child.

#### **Conclusion**

When counseling patients who are considering advanced reproductive technologies to conceive their child(ren), it is important to consider the health and age of the couple, genetic conditions, as well as previous fertility and pregnancy indicators. While the majority of children will be healthy, the risk of adverse outcomes increases with genetic predisposition, advanced maternal and paternal age, and severe infertility.

Couples should consider and weigh all possible options for having a family in light of the risks and benefits of infertility treatment.

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## How is male-factor subfertility minimized in companion and food-producing animals?

R.P. Amann

### Introduction

Propagation of companion and food producing animals is primarily by planned use of sires known to transmit desired traits. Exclusion of males likely to be noticeably subfertile is also a goal. Use of superior sires is facilitated by artificial insemination (AI) (Chapter 25). AI predominates with dairy cattle and pigs, is popular with dogs and horses, but is banned for thoroughbred horses. Natural mating predominates with widely dispersed beef cattle and sheep. Few unselected dairy bulls have an observed pregnancy rate >8% below the national average, because for >15 generations bulls with inferior semen or low pregnancy rate have been culled before extensive use via AI. However, 1 in 5 unselected beef bulls might be subfertile.

### Reproductive function

Species differences are superbly described and illustrated in Senger's text. Testes hang between the hind legs, except in pigs where they are below the anus. Stallions have an erectile penis, like a human, whereas bulls, boars and rams have a fibroelastic penis which straightens, from a non-erect S-shaped structure, on engorgement with blood. In the dog, an os penis allows intromission before complete vascular engorgement. Except for dogs, the complement of accessory sex glands is as in humans, i.e., seminal vesicles, prostate, and bulbourethral glands.

Spermatogenesis requires 39 to 61 days, depending on species. Daily sperm production per gram testis parenchyma ( $10^6$ ) typically is 10–19 in bulls, dogs or stallions and 21–25 in boars or rams (contrasted to  $4 \times 10^6$  for men). Weight of a single testis might range from 8 to >700 grams (small dog; boar) and daily sperm production per male ( $10^9$ ) ranges from near 0.4 for dogs; 5–8 for bulls, rams, and stallions; to 16 for boars (contrasted to 0.07–0.28 for men). Transit of sperm through the epididymal duct takes 7–17 days, with most of this interval spent within the cauda epididymidis. Paired caudae epididymides can accommodate sufficient sperm ( $3\text{--}130 \times 10^9$ ) for a number of ejaculates. Frequent ejaculation reduces number of sperm in the caudae

epididymides, by up to 50% when  $\geq 10$  ejaculations occur on 1 day. With no emission/ejaculation over 6–10 days, sperm “spill out” into the pelvic urethra and are voided with urine.

Endocrine regulation of reproductive function in male animals is similar to that described for men (Chapters 2,4,5,8). However, there are both diurnal and seasonal variations in pulsatile secretion of LH and testosterone. Seasonal variation is pronounced in rams and stallions, and sperm production declines 30–80% in the non-breeding season (fall and spring, respectively in northern latitudes). Photoperiod is the primary signal synchronizing an endogenous rhythm.

### Semen collection

Examination of sperm is central in minimizing subfertility. Semen collection from most animals is easy using an artificial vagina (AV), masturbation, or electroejaculation (EE). Motility and morphology of sperm are not affected by method of semen collection. An AV is a cylinder, which contains warm water between the jacket and latex liner to provide warmth and slight pressure on an inserted penis, and an attached container to receive semen. An AV can be used with males of most species. The male is provided with a teaser animal or dummy and when he mounts the penis is directed into the AV allowing intromission and ejaculation. This approach usually provides a representative ejaculate. Semen from stallions and bulls usually is collected with an AV. With boars and dogs, penile pressure stimulates ejaculation. A male is allowed to mount an estrous female or phantom and the collector simply grasps the free end of the protruded penis to mimic the interior of a pig's cervix or a dog's vaginal vestibular muscles, applies necessary pressure, and directs semen into a receptacle. EE involves appropriate restraint of a male, transrectal placement of a probe with 3 longitudinal electrodes over adjacent nerves, and rhythmic application of mild electrical stimulation. This method is useful with bulls, rams, and wild animals. The resulting ejaculate usually is more dilute than one obtained using an AV.

Emission and ejaculation require only a few seconds in bulls and rams, or <1 minute in stallions. In boars and dogs the ejaculatory process is a minutes-long series of emissions and ejaculations, so it is common to direct only the sperm-rich fraction into the semen receptacle. For boars, bulls, dogs, rams, and stallions a spermatozoon has a paddle-shaped head with a compact acrosome over the rostral portion of the nucleus, under the plasma membrane. The shape of the head allows distinguishing sperm from one or another species.

## **Breeding-soundness examination**

Owners of food-producing and companion animals know that most males will not be needed as sires, because one male can impregnate >25 females during a breeding season lasting 1–3 months (beef cattle, horses) or throughout a year (dairy cattle, dogs, pigs). Future sires are selected for genetic reasons and unselected males are castrated. After puberty, selected males usually are given an andrological or breeding soundness examination (BSE) by a veterinarian.

The goal of a BSE is to identify males likely to be subfertile, or for other reasons unsuitable for breeding, at the time of examination. Subfertile is a relative term, and for companion and food producing animals might be a male with relatively small testes or whose semen has more immotile or abnormally shaped sperm than desired. A BSE includes a history; complete physical examination; evaluation of the testes, epididymides, accessory sex glands, and penis; and collection and evaluation of semen. The number of sperm in an ejaculate (considering age and species) and the motion and morphologic characteristics of sperm collected can be diagnostic, especially if multiple samples are collected. Absence of sperm in an ejaculate does not mean the male is sterile, because occasionally emission of sperm from the excurrent ducts will not occur despite emission of accessory sex gland fluids which then are ejaculated.

In good quality semen, >70% of the sperm have a normal head shape, a non-swollen acrosome, no residual “droplet” of cytoplasm in the neck or annulus region, and have a normal looking tail. Especially with boars and bulls, specific morphologic defects have been linked with inability of a spermatozoon to fertilize an oocyte or to produce a normal embryo. In good quality semen, >60% of sperm should display progressive motion when examined in a diluted suspension at 37°C.

A male failing a BSE might pass a similar exam 1-6 months later. Passing a BSE often is a prerequisite for sale of a sire. A male passing a BSE likely will be of reasonable fertility, i.e., commercially useful, when mated with normal females. However, subsequent changes or factors not detected might depress his fertility. An example is lack of libido under range conditions, because a test of serving capacity is not part of a BSE and EE usually is used to obtain examined semen. Importantly, when a sire is used with a group of females the collective pregnancy rate will depend on female and management factors.

As breeding progresses, animal owners check females for pregnancy. If an owner suspects pregnancy rate is low in a natural mating situation, a veterinarian might be asked to perform a BSE on the male(s). This is a frequent occurrence with valuable stud dogs, stallions, or beef bulls. Then

issues include: “Why is pregnancy rate, semen quality, or sexual behavior of this male poor?” and “What is the prognosis for improvement?” Usually, the passage of time (>2 months) is the most cost-effective therapy. Genetic companies monitor quality of semen prepared for sale, in an integral andrology laboratory, and also outcomes with semen sold for AI. They cease distribution of any male whose semen quality drops below their standards or with a pregnancy rate that seems low, e.g., ≥8 percentage units below average.

## **Factors associated with subfertility**

Clinical problems in previously normal animals arise from several causes. Perhaps most common are heat and humidity. Heat affects many boars, bulls, and stallions. To avoid a “summer slump” in semen quality, i.e., decreased percentages of morphologically normal or motile sperm due to elevated intratesticular or cauda epididymal temperature, many breeders or genetics companies house valuable males in air-conditioned facilities. Rams are less affected because they are fall breeders and their testes are “ramping up” as ambient temperatures decline. Stud dogs usually are kenneled in cooler areas. Nevertheless, cases of oligozoospermia and increased numbers of morphologically abnormal cells consequent to heat-stress are seen. Cool temperatures usually are not a problem. Temperature-induced changes are diagnosed by history or BSE results. Therapy involves eliminating the causative factor and allowing 2-6 months for recovery.

Infectious agents are common problems, especially venereal diseases. Brucellosis is a venereal disease that causes epididymitis, orchitis and azoospermia in cattle, sheep and dogs. Affected animals should be euthanized. Other bacterial infections are common in stallions and stud dogs. White blood cells in semen cause suspicion and differential semen-culture-based tests identify the organism. Therapy is as indicated by sensitivity in culture.

Trauma is a common problem in free-ranging sires. Diagnose is easy and treatment involves surgical intervention or medical therapy. Parasitism and metabolic problems are uncommon, but can be diagnosed from history and clinical presentation which then guide medical treatment.

## **Summary**

Male-factor subfertility is minimized by selection via andrological examinations and follow-up evaluations of ejaculate and sperm characteristics. Males with small testes or producing inferior semen, whose sperm display

sub-standard quality in laboratory evaluations, or whose semen does not provide a commercially useful pregnancy rate, are eliminated from a breeding program.

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## **What should I know about artificial insemination (AI) of animals?**

R.P. Amann and D.L. Garner

### **Why does AI have great impact?**

Greater numbers of superior offspring is the major objective of AI. Secondary advantages include virtual elimination of disease transmission during breeding and reduction of human injuries from dangerous large animals. With natural mating 1 boar, bull, dog, ram, or stallion can impregnate >25 females in 1–3 months. With AI up to 25,000 cows or almost 100 pigs can be bred with 1 week's production of sperm from a male. Hence, with AI most males are not needed to produce future generations, so sire selection is intense. Potentially superior sires are produced by planned use of semen from a male with many progeny displaying outstanding production traits.

AI facilitates intensive paternal selection. This has markedly improved milk production per cow and provided lean and tender carcasses in swine remodeled in <10 generations. A few bulls have produced >150,000 insemination doses in a year or 1.5 million doses in their lifetime. Spreading costs among many insemination doses allows rigid health control of AI sires, special housing, quality control in the andrology and processing laboratories, monitoring of reproductive performance, and transmission of desired traits to offspring.

Most boars in an AI program are used extensively starting around 7 months of age and are replaced ≤12 months later. With dairy cattle, semen processed from 11–15 months of age is used to inseminate ~1,500 females in 75–150 herds. Up to 10,000 AI doses might be used from an occasional elite young bull. The bull then is held from use until the quality of his female progeny is established by phenotypic evaluation for actual ease of calving and production of milk protein after an additional year. Daughters of a given sire are compared with those of all other sires used in the same and other herds to determine which sires best transmit desired traits. Approximately 1 of 9 “evaluated” sires is returned to active use and the other 8 are eliminated. By 2012, analyses of single nucleotide polymorphisms should allow meaningful estimation of a new born female's genetic potential for facile calving and milk production. Such data for a bull's first 200 female calves will shift the paradigm for sire selection, especially as accuracy of predictions is refined. Currently, a typical AI bull might remain in service for 3–5 years. Dogs and stallions remain in use as long as there is demand for their semen.

AI usually is performed by transcervical intrauterine deposition of sperm. After ejaculation, sperm are extended in special medium and held at 5°C (most species) or 17°C (pigs) for use ≤6 days after ejaculation or cryopreserved and maintained at -196°C for use over months or years (Chapter 14). This allows national distribution of liquid semen to inseminate females and both national and world-wide distribution of frozen semen (consistent with international biosecurity and health regulations). Liquid semen dominates with pigs, because it efficiently utilizes sperm where thousands of sows are nearby; procedures for cryopreservation remain imperfect for this species. Both liquid and frozen semen are used with dogs and horses (AI banned for thoroughbred horses). Frozen semen dominates with dairy cattle. Frozen semen is used with beef cattle, although natural mating predominates. AI with liquid semen is obligatory with commercial turkeys as males become too large to mate before reaching puberty. In every case, proper semen handling is crucial.

### **Use of AI**

Approximately 11 million cattle are inseminated annually in North America and 11 million in Europe. Worldwide, approximately 230 million doses of frozen semen and 12 million doses of liquid semen are prepared annually, with 2–3 doses required to impregnate most females. Most pigs are inseminated with liquid semen, totaling 21 and 18 million AIs annually in North America and Europe.

### **How is outcome from AI measured?**

Fertility simply means “being fertile”. Conception rate or fertilization rate is the proportion of oocytes exposed to sperm that form a zygote. Because in vivo fertilization rate cannot be measured except in special research projects, it has limited value. Pregnancy rate is useful provided the mean value is accompanied by the measure used to determine pregnancy, e.g., ultrasonic detection of embryos, and the interval after mating or insemination when the observations were made, number of females contributing to the mean value, and a statement if the percentage was calculated for all females eligible for breeding or only females actually bred. Pregnancy rate always will be lower than fertilization rate because some zygotes will not develop into detectable embryos or viable fetuses.

It is common to refer to subfertile or highly fertile males, based on pregnancy rate obtained with a population of females. However, this overlooks 3 important facts. First, the male might be penalized for lack of pregnancy in



females that never were bred. Second, first mating pregnancy rate will be lower than cumulative outcome at the end of a breeding season. Third, observed pregnancy rate = [male's fertility]/[(female's fertility)(management factor)]. The management factor includes insemination of animals not in estrus. Validity of this "equation" is best evidenced by extensive data showing that semen from a male gives very different pregnancy rates when used with nuli-parous vs. parous females or with females in different producer units.

### **Modifying sperm before AI**

Animal andrologists learned >65 years ago that one can improve on nature. Exposure of animal sperm to certain lipoproteins or proteins, rather than dilution in a simple salts solution, improved retention of membrane integrity and increased pregnancy rate with AI. Thousands of publications describe improved "extenders" and procedures. Usually they targeted improved function or survival after storage at 17°C, 5°C, or -196°C. Additives include antioxidants, vegetable-derived molecules, and synthetic molecules based on molecules found in semen.

### **Use of "sexed sperm"**

With food producing animals there can be substantial economic benefit from modifying sex ratio at birth. Females might be preferred in dairy cattle and pigs operations. The concept started to move from dream to reality in the 1980s with development of flow-sorting instruments capable of detecting the 3–5% greater amount of DNA in X-chromosome-bearing sperm than in Y-chromosome-bearing sperm. Research to bias sex ratio based on other attributes of sperm continued.

Moving flow-sorted sperm to a commercial commodity required that the patent holder solve myriad electronic, computer programming, fluidic, and biological problems. By 1999 progeny of the desired sex, for several species, were being produced with regularity. Technical improvements enabled establishment of commercial flow-sorting facilities in the UK in 2000 and USA in 2004. Starting in 2006, flow-sorters were positioned at major cattle genetics companies. Worldwide, by late 2008 there were sorters in 17 commercial facilities. Sorter throughput limits an AI dose to  $\sim 2 \times 10^6$  sperm and possible suppression of pregnancy rate is minimized by AI primarily of nuli-parous females. More than 60% of sperm are discarded during sorting because they are of the wrong type, ambiguous to the machine, or dead. Desired sperm are processed, packaged into 0.25-mL straws, and cryopreserved. Approx-

imately 90% of sperm in a commercial dose of flow-sorted semen have the desired sex chromosome.

Three million doses of sex-sorted cryopreserved semen were produced at 5 sites in USA in 2008, with  $\sim 1$  million of these exported. To date,  $\sim 7$  million doses have been produced in USA and another 3 million doses in other countries. Approximately 97% of production has been for X-chromosome-bearing sperm, and producers obtain  $\sim 90\%$  female offspring. In a recent report, 47% of 33,879 nuli-parous females conceived after 1 insemination; this pregnancy rate was 80% of that after AI of conventional semen ( $15\text{--}20 \times 10^6$  sperm) into other nuli-parous females in the same 157 herds.

### **Summary**

AI was the first widely used technique for assisted reproduction and remains the most important for genetic improvement and production of newborn. In the USA, millions of dairy cows, pigs and beef cows are bred via AI, as are fewer horses. Flow-sorting sperm to predetermine sex at birth works with many species, but is a commercial reality only with bull sperm; 5 million cryopreserved doses produced in USA to date.

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## Is there a decline in sperm counts in men?

R.J. Sherins and G. Delbès

Over a number of decades now, there has been continuing global concern regarding the potential for diminishing human reproductive health in industrialized countries. This has been supported by the discovery of congenital genito-urinary defects among some lower species exposed to industrial toxicants in the environment. Of particular concern were the “estrogen-mimics” (xeno-estrogens) because of their potential to disrupt cellular and physiological functions, even in minute doses. This gave birth to the concept that exposure to such “endocrine disruptors” could produce clinically detectable changes in human reproductive functions; in-utero and early exposures being the potential cause of long term male reproductive disorders.

The credibility of this hypothesis was supported by a 1992 meta-analysis of 61 studies of human semen quality published during a 50 year period (1938-1991), representing almost 15,000 men from among 23 different countries; an approximately 50% decline in sperm counts (113 to 66 million/ml) was found over that time frame. Further concern about a temporal (secular) decline in semen quality was heightened by a 1995 French publication that reported a 30% decrease in sperm counts (89 to 60 million/ml) from among Parisian sperm donors during a 20 year period (1970-1992). Thereafter, a large number of additional studies, reviews and editorials ensued to support or reject the concept that “male fertility”, as measured by sperm count, was in fact changing. However, among the aggregate research reports, only a few showed unambiguous declines in semen quality; most studies found no decline, an increase or mixed changes in semen parameters (Chapter 12). Nevertheless, the worrisome concern that human “male fertility” was diminishing was kept in the headlines primarily by the media that focused on only a portion of the data (declining sperm counts); while the scientific community raised substantive questions about the validity of the data as detailed below.

Among the many reported studies, criticisms regarding the conclusions in some papers that there are global secular changes in semen quality are based on numerous issues known to profoundly affect semen quality. These include: 1) lack of standardized criteria for semen sample collection, 2) bias introduced by using different counting methodologies, 3) inadequate within-individual semen sampling in the analysis, 4) failure to account for variable abstinence intervals and ejaculatory frequency, 5) failure to assess total sperm output rather than concentration, 6) failure to assess semen

parameters other than the number of sperm, 7) failure to account for age of subject, 8) subject selection bias among comparative studies, 9) inappropriate statistical analysis, 10) ignoring of major geographic differences in sperm counts, and 11) the casual equating of male fertility with sperm count per se.

Unlike the tight homeostatic regulation of blood component levels in the internal milieu, semen parameters vary considerably because the ejaculate is an excretory product that is influenced by many external conditions. Ejaculatory frequency and abstinence interval are major determinants of sperm output and semen quality. With prolonged intervals between ejaculations (days), sperm output increases due to higher caudal epididymal reserves, but motility and normal morphology values decrease. Seminal fluid volume is also highly variable between and within individuals; thus, only total sperm output adequately quantifies sperm number in the ejaculate. In addition, it is well known that even under standardized conditions of semen collection there is marked variability of within-individual sperm counts (75% coefficient of variation), such that at least three semen samples are generally required to obtain a stable estimate of semen parameters. Thus, retrospective studies that assess only one semen sample collected under non-standardized conditions are seriously flawed. In addition, semen quality diminishes considerably with advancing paternal age. Since the age of subjects reported in the various studies extends over many decades, comparisons of such data between groups must be done with great caution.

Over and above the technical concerns regarding interpretation of the reported semen analyses, subject selection bias among the studies appears to be an even more critical issue. Within some publications, study subjects included younger sperm donors, older pre-vasectomy patients or patients attending infertility clinics. None of these groups are representative of the general population, and within each subject group, selection criteria are not defined and may have varied significantly over the time frame of the study.

An additional factor that has yet to be adequately explained is the marked difference in sperm counts reported from differing geographic regions even within the same country. An inclusive analysis of all 29 available reports of sperm counts from only the United States during almost 60 years (1938-1996) revealed a statistically significant secular decline. However, sperm concentrations from the four publications involving subjects living only within New York cities were significantly higher (30%) compared to all other U.S. cities and there was no statistical difference in values reported from among the four New York cities. When the New York data were eliminated from analysis of the remaining 25 studies, no temporal decline in sperm counts was evident. Most critically, re-analysis of the 1992 meta-analysis of 61 studies of sperm counts revealed that no secular decline was

evident when the four earlier New York data sets were eliminated.

Given the above discussion, it seems impossible at present to scientifically conclude that there is or is not a worldwide secular decline in human sperm counts or male fertility. However, the regional differences in sperm counts have yet to be explained and deserve further investigation. In addition, assessments of sperm quality other than count, motility and morphology need consideration as a measure of male fertility potential. Recent studies of sperm chromatin structure may be relevant. Concerns that environmental toxicants, as well as obesity and lifestyle changes, may be impacting human reproductive health are likely to be important considerations. Support for such concerns comes from parts of Europe where there is now evidence for an increasing incidence of testicular cancer, and preliminary evidence for congenital genito-urinary abnormalities (hypospadias, possibly cryptorchidism) as well as secular, age-independent decline in serum total testosterone and sex hormone binding globulin in both age-matched Danish and American men. The shared risks for these testicular disorders have lead to the concept of a “Testicular Dysgenesis Syndrome” that might include downstream changes in semen parameters (Chapter 33).

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## How do environmental cues affect male reproduction?

W.W. Wright

### The effect of season on reproduction

Reproduction is energetically expensive. While the physiological cost is primarily born by the mother, males of many species use considerable energy both to mate and to aggressively prevent other males from mating. As an adaptation to these high energy costs, mating of many species is restricted to a specific mating season. The timing of this season insures that females have a maximal food supply during the most energetically expensive periods of late pregnancy and lactation. For example, wild sheep living in Northern latitudes breed in the fall and lambs are born in the spring. For males, a breeding season restricts their reproductive energy costs to the time of year when females are fertile. The onset of the breeding season has specific physiological underpinnings and in the male, it is associated with activation of the hypothalamic-pituitary-testis axis (Chapter 2). Thus, the synchronization of this axis to specific environmental cues is important to male reproduction in many species.

Obviously, modern humans are not seasonal breeders. Nonetheless, there is evidence that environmental cues play a role in our reproductive biology. In the Southern United States, numbers of births is highest in July through September and lowest in March through May. Thus, conceptions peak in October through December. This seasonal reproductive cycle is less pronounced in the more Northern of the United States, indicating a diminution in the intensity of the environmental cues affecting human reproduction. In contrast, in Finland, Denmark, Austria, Syria and England number of births peak in January through April. However, over the past 500 years, there has been a marked diminution in the seasonal changes in English birth rates. This suggests that humans' increasing control over their living environments is reducing the impact of natural environmental cues.

The three relevant environmental cues are day-length, temperature and availability of food and water. The importance of specific cues undoubtedly varies between different geographic locations. In more Northern latitudes, day-length changes markedly and humans living North of the Arctic Circle experience days of constant darkness in the winter and days of constant light in the summer. In the tropics, day length is essentially constant but seasonal changes in rainfall can have a profound effect on food supply of some indigenous peoples and consequently, their reproductive function. In the Southern United States, high ambient summer temperatures may be

responsible for the trough in conceptions that occurs in June, July and August.

### Seasonal changes in male reproductive function

There are major changes in serum gonadotropin levels and testis function of seasonally breeding animals. In male Rhesus monkeys, serum LH and testosterone levels are increased 4 to 5-fold during the winter breeding season and testis size and sperm production are doubled. Other species show even greater seasonal changes in testis function. For example, with completion of the breeding season for Roe deer, sperm production gradually decreases to zero and testosterone levels fall by more than 95 percent.

There is also evidence that season influences serum levels of LH and testosterone of men. One study reported that in Seattle, the serum concentrations of testosterone and LH in men were highest in June and July and lowest in August and September. In contrast, serum LH levels in Norwegian men dipped in December and this seasonal decline was greater in men living North of the Arctic Circle. However, serum testosterone levels in Norwegian men were not reduced in December, emphasizing the disparate effects of seasonal cues on reproductive function of the human male.

### Melatonin as a mediator of day-length on male reproduction

As noted above, a substantial change in day-length is a potentially important environmental cue for seasonal breeders. These cues are first detected by the retina and then interpreted by the circadian pacemaker of the brain. A series of nerves connect this pacemaker to the pineal gland, which in humans is found between the cerebral cortex and the cerebellum. The pineal transduces day-length information from the pacemaker by altering its diurnal secretion of the hormone, melatonin. In seasonally breeding animals, melatonin secretion is suppressed by long days and changes in serum melatonin levels can significantly affect reproductive function. However, the nature of the effect depends on whether the animals breed in the winter or in the spring and summer. For example, when male arctic foxes, who breed in the winter, were implanted with melatonin pellets during the arctic summer, the initiation of the winter rise in serum testosterone levels was advanced by two months and the testicular regression that normally occurred with the end of the breeding season was blocked. In contrast, melatonin caused regression of testes of Djungarian hamsters that were simultaneously exposed to stimulatory effects of long day-lengths. The mechanism of action of melatonin in mammals has not been completely defined. However, in

specific experimental models, melatonin can increase GnRH secretion, suppress LH secretion or suppress gonadotropin-stimulated steroidogenesis.

In contrast to what has been established for seasonally breeding animals, a role for melatonin in seasonal changes in human male reproduction has not been established. Changes in day-length affect the secretion of melatonin in only a subset of humans and to date, there has been no convincing evidence that a sustained increase in serum melatonin levels affects serum LH or testosterone levels in men.

### **Heat has a direct inhibitory effect on spermatogenesis**

Elevated temperatures are deleterious to sperm production. Heating the human scrotum to 43°C for 30 minutes was shown to cause a significant increase in germ cell death and an 80% decrease in the numbers of sperm in the ejaculate. Conversely, a 12-week course of overnight scrotal cooling was reported to increase numbers of sperm in the ejaculates of men with otherwise low sperm counts. The deleterious effects of heat have also been noted in retrospective study of bakers and welders where it was concluded that occupational heat exposure was a risk factor for human male infertility. Tight underwear, riding a bicycle and sitting with a lap top computer on one's lap all increase scrotal temperature and thus, potentially, the temperature of the testis.

### **Summary**

Environmental cues affect reproductive capacity of males of a number of species. Seasonal changes in numbers of human births suggest that such cues can also affect the reproductive biology of men. The dramatic changes in day-length experienced in more Northern latitudes and prolonged elevated temperatures experienced in Southern latitudes as well as in some industrial work places may affect serum levels of essential reproductive hormones as well as the production of viable male gametes.

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## **What are the effects of environmental toxicants and/or endocrine disruptors on sperm function?**

*Chemical exposure effects on the male,  
reactive oxygen species and spermatozoa*

S.D. Perreault

### **What is the evidence that environmental contaminants may impact sperm function in humans?**

Our appreciation for the potential impact of chemicals and other environmental contaminants on male reproductive function (spermatogenesis and fertility) was awakened in the 1970s when a group of women whose husbands worked in a plant manufacturing the fungicide DBCP (dibromochloropropane) discovered that they were all having trouble getting pregnant. Subsequent epidemiological investigations showed an association between the men's exposure to DBCP and low sperm counts or even azoospermia. Furthermore, sperm production improved in most men when the exposure ceased. This rather serendipitous finding raised awareness that infertility was not necessarily a female problem and motivated regulatory agencies like the US EPA (Environmental Protection Agency), USDA (United States Department of Agriculture) and FDA (Food and Drug Administration) to improve their testing procedures and protocols in order to detect potential male reproductive toxicants. It also motivated NIOSH (National Institute of Occupational Safety and Health) and others to conduct occupational health studies for a variety of chemicals. We now know that in addition to certain pesticides, many environmental contaminants including certain industrial solvents, plasticizers and metals (notably lead and cadmium) have the potential to disrupt spermatogenesis and or specifically alter sperm function, given a sufficiently high exposure.

In general, evidence for adverse reproductive effects in humans has been found only in small cohorts of men exposed to relatively high levels of chemicals in occupational settings or from accidental chemical release. The extent to which the low levels of compounds or combinations of compounds that we encounter in ambient day-to-day environments may impact male reproduction is largely unknown and far more difficult to determine.

## **How do regulatory agencies test chemicals for spermatotoxicity and predict male reproductive effects?**

In 1998, the US EPA updated its testing protocols required for the ([http://www.epa.gov/opptsfrs/publications/OPPTS\\_Harmonized/870\\_Health\\_Effects\\_Test\\_Guidelines/Series/870-3800.pdf](http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-3800.pdf)) registration of pesticides. The current guidelines, that have been harmonized across federal agencies in the US and internationally, call for multigenerational testing in animal test species (primarily rats) where measures of sperm production and function (sperm motility and morphology) are obtained in parental adult animals and in their offspring. The protocol also evaluates reproductive tract development after *in utero* or early life exposures. Because males are dosed for the entire duration of spermatogenesis before they are evaluated and bred, adverse effects on any spermatogenic or somatic cell target should be detectable in the form of reduced testis or epididymal weights, altered testis histology (one of the most sensitive indicators of effects), reduced epididymal sperm counts, altered sperm motility or morphology and/or reduced fertility. When effects are seen, additional specific tests for sperm function such as *in vitro* fertilization (IVF) and evaluation of sperm chromatin structure, DNA damage, and chromosome numbers/structure can be used to further characterize the effect (Chapter 13).

Although typically not required for non-pesticidal industrial chemicals, these standard and specialized tests are being applied on a voluntary basis to evaluate chemical safety for compounds produced and used in high volume and those predicted to have reproductive effects based on their similarity to known toxicants (e.g. by structure-activity analysis) or based on *in vitro* screening for endocrine activity, oxidative stress, mutagenicity or cytotoxicity.

Regulatory agencies consider all available information from such tests when they perform a risk assessment, the complex process that integrates dose-response data from the test species, the chemical's mode of action (if known), the relevance of the animal data to humans, and any available human epidemiological data with the goal of predicting risk to humans. In addition to formal risk assessments conducted by regulatory agencies, the National Toxicology Program conducts reviews of high priority reproductive toxicants through its Center for Environmental Risks to Human Reproduction (CERHR) and posts reports on the internet (<http://cerhr.niehs.nih.gov/>). CERHR selects chemicals based on both their occurrence (production volume and likely human exposure) and mode of action (likely reproductive

toxicant), thereby evaluating compounds of high public and regulatory concern such as acrylamide, bromopropane, phthalates and bisphenol A.

### **Biologically plausible modes of spermatotoxicity: Oxidative stress**

Sperm are unique in their metabolism and actually use oxidative stress to advantage. Reactive oxygen species (ROS) generated by metabolically active sperm play a role in the normal signaling that leads to capacitation or the capability of fertilizing eggs (Chapter 15). However, excess ROS can also cause membrane damage (lipid peroxidation) and sperm DNA damage, with undesirable consequences on fertilizing ability and genetic integrity of the ensuing embryo, respectively. Therefore, environmental contaminants that generate oxidative stress or alter REDOX status (including DBCP, acrylamide and bromopropane) are biologically plausible candidate spermatotoxicants. An understanding of oxidative stress in sperm is also directly applicable to assisted reproductive technologies. Because sperm can undergo oxidative damage during *in vitro* culture, media components and IVF procedures are being optimized to minimize such effects.

Few known male reproductive toxicants act only or specifically on sperm maturing in the epididymis. Alpha-chlorohydrin, a sperm metabolic poison that inhibits sperm motility, is one such chemical. However, some toxicants can have direct effects on sperm motility and/or fertilizing ability and impair earlier stages of spermatogenesis. For example, chronic exposure to acrylamide can damage spermatogonia and spermatocytes and indirectly impair fertility by arresting spermatogenesis. At levels that do not arrest spermatogenesis, the reactive metabolite of acrylamide can also alkylate sperm nuclear protamine during the final stages of spermiogenesis. As a result, the sperm may be able to fertilize eggs, but the resulting embryos die due to acrylamide-induced damage in the sperm pronucleus. The good news is that effects of chemicals that react directly with maturing sperm are as transient as the exposure. Once the exposure has ended and the affected sperm have been released from the body, the risk disappears.

Cigarette smoke and air pollution resulting from the combustion of coal and gasoline contain reactive chemicals that can cause oxidative stress and inflammatory changes in the respiratory and cardiovascular systems. Both cigarette consumption and exposure to high levels of air pollution have also been associated with altered semen quality and DNA damage in sperm. Because such sources of oxidative stress can be difficult to avoid, antioxidant dietary supplements may be of therapeutic or protective value. However, convincing benefits of this strategy have been hard to measure in human studies.

### **Biologically plausible modes of spermatotoxicity: Endocrine disruptors**

Certain chemicals may impair sperm function by interfering with androgen action. For example, toxicology studies in rats show that the fungicide vinclozolin (and its metabolites) inhibits androgen action by binding to the androgen receptor, and some phthalates interfere with steroidogenesis resulting in reduced testosterone synthesis. Some chemicals such as bisphenol A can act as estrogens, while others are promiscuous and bind multiple receptors. Because hormones themselves are active at very low levels, these so-called “endocrine disruptors” have the potential to impact human reproductive processes, even when present at the low levels typically encountered in the environment. Furthermore, the developing male reproductive system is particularly sensitive to altered testosterone signaling. Toxicology studies in rats, where specific windows of exposure and sensitivity can be precisely defined, have shown that blocking androgen action during reproductive tract development can result in hypospadias and testicular maldescent in male offspring. Since the later stages of spermiogenesis depend on androgen, and there is increasing evidence for a role for estrogen in sperm function, endocrine disruptors may also impact sperm function in adults. At environmental levels of these contaminants, such effects are likely to be subtle and difficult to detect. However, even subtle effects may have negative impacts on subfertile men who are thought to be more susceptible to environmental stressors.

Based on growing evidence about the occurrence and potentially adverse effects of environmental endocrine disruptors, regulatory agencies have been directed by Congress to develop an effective screening and testing program for contaminants with endocrine disruption potential (<http://www.epa.gov/endo/>). These screening tests and other high throughput test systems currently under development will make it more feasible to test the large numbers of new chemicals introduced into our environment by commerce and thereby protect our reproductive health.

### **How can clinicians advise their infertility patients and couples attempting pregnancy?**

Clearly there is a need for patient-oriented information about the linkage between environmental contaminants and risks to male reproductive function. The Collaborative on Health and Environment makes such information readily available on the web ([http://www.healthandenvironment.org/working\\_groups/fertility](http://www.healthandenvironment.org/working_groups/fertility)). At present, physicians can at least include a

short history on occupational and environmental exposures and advise their patients to avoid exposures to known toxicants such as cigarette smoke, solvents and pesticides.

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**Can spermatozoa be targets for drugs? If so, what are the consequences of such drug exposures? Is there a need for pre-conception counselling for men?**

*Drugs that affect sperm structure or function, male-mediated developmental toxicity, prevention, tests to detect damage to spermatozoa*

B.F. Hales and B. Robaire

There are more than 100,000 chemicals in our environment. Men are exposed to chemicals in air, water, and food, in daily life, in occupational settings, and as drugs. Some of these chemicals do target spermatozoa (Chapter 28). Chemicals may have adverse effects on male germ cell numbers, decreasing production by blocking mitosis and/or meiosis, or increasing cell death by inducing apoptosis, an active process by which cells commit suicide. Alternatively, chemicals may affect germ cell quality, without an accompanying effect on sperm counts. Finally, chemicals may disturb the male germ cell genome, by mutating the DNA sequence itself, or alter the epigenome (Chapter 9), by affecting modifications to the DNA bases or to the proteins that make up male germ cell chromatin.

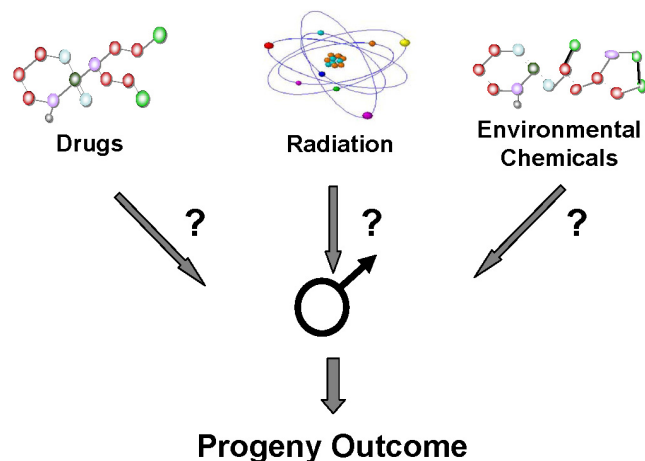


FIG. 1. Environmental factors and xenobiotics can affect male germ cells with consequences for future generations.

Male germ cells are engineered to fertilize an oocyte and to provide a paternal genome to the conceptus; chemicals that target male germ cells may decrease their fertilizing ability or induce post-fertilization adverse effects on progeny outcome. The success of in vitro fertilization techniques is a direct measure of fertilizing ability. In vitro fertilization technology is well established in humans and certain animal models, such as mice, but less successful in other common lab species, such as rats. An in vitro fertilization assay with rat zona-free oocytes revealed that sperm fertilizing potential was reduced by treatment of rats with toxicants, such as m-dinitrobenzene and ethylene glycol monomethyl ether, that did not affect sperm motility. Although there are very few human studies, in IVF-treated women there was no increased risk of spontaneous abortion attributable to paternal exposure to welding or pesticides. When other parameters that do not distinguish between an effect on fertilizing ability and early pregnancy loss, such as cycle-specific fertility rates, time to pregnancy, cumulative percent pregnant, and fecundability are assessed, effects have been reported after paternal exposures to common environmental toxicants, such as lead.

Adverse progeny outcomes might include early or late pregnancy loss, preterm delivery or delivery of a small-for-gestational age infant, malformations, behavioral abnormalities, or even childhood cancer. There is convincing evidence from animal studies that paternal exposures to specific environmental or therapeutic agents do result in a higher incidence of adverse progeny outcomes. A wide range of environmental chemicals (e.g., lead, dibromochloropropane, phthalates) and drugs (e.g., the anticancer alkylating agent, cyclophosphamide) produce abnormal progeny outcomes after paternal exposure. From the timing between toxicant exposure and the effect on offspring, one can deduce the stage specificity of the susceptibility of germ cells during spermatogenesis to damage.

A number of epidemiology studies show that certain paternal occupations, e.g. as a welder, painter, auto mechanic, greenhouse worker or fireman, involving exposure to metals, combustion products, solvents, or pesticides, are associated with altered sperm quality, and an increase in time to pregnancy, spontaneous abortions, birth defects, or childhood cancer. Life style exposures, such as paternal smoking, have been linked to an increased incidence of childhood lymphomas. Therapeutic drug exposures may also be of concern with respect to progeny outcome. After men are treated with anticancer drugs, there is a high incidence of transient or permanent infertility. The extent to which the sperm produced during recovery from chemotherapy are "normal" deserves further investigation. However, when these men have fathered children, the proportion of malformed children has not been higher than in control groups.

It is very difficult to associate a paternal exposure with a specific birth defect or childhood cancer since the numbers in the studies are normally low, exposure assessment methods are frequently imprecise, and the outcomes are rare; thus, the lower limits of the confidence intervals surrounding the risk estimates are often less than half a unit away from the null value. One should not automatically dismiss cause-and-effect relationships only on the grounds that they are small.

It is of concern that the germ cell line of progeny may be affected, thus increasing the risk for subsequent generations. Generally, the basic premise has been that the chemicals that are capable of affecting subsequent generations do so by inducing mutations in the germ cell genome. Studies with human populations and mice have provided evidence that paternal irradiation exposures result in elevated mutation rates in progeny. However, in recent years it has become evident that the male germ cell transmits more than its genome, as specified by DNA sequences, to the conceptus. In animal studies, there is a large gap between the generally low rate of genetic “damage” induced by many chemicals after the exposure of male germ cells and the associated adverse progeny outcomes. This gap has led to the hypothesis that a key mechanism by which chemical perturbation of sperm may lead to heritable alterations in progeny is epigenetic, i.e. the alteration of DNA function without affecting DNA sequence, regulated by modifications to histones (Chapter 9). Deregulation of the programming of the paternal genome may be responsible for altered expression of genes and impaired post-implantation development.

Future research is needed to elucidate the implications to public health of the finding in animal experiments that chemical exposures may have transgenerational effects. Nevertheless, there is already suggestive evidence from human studies indicating that this is possible. There are reports of altered sex ratios in children born after exposures to chemicals such as dioxins; one explanation for these observations could be an effect on the survival of Y bearing sperm. Defects in DNA methylation of paternally imprinted genes may contribute to imprinting errors and disease in children conceived with assisted reproductive techniques (ART), perhaps due to the in vitro culture conditions.

It is apparent from both animal and epidemiological studies that there are paternal exposures to chemicals that result in abnormal progeny outcome. Men exposed to certain chemicals because of their occupation should be made aware that there is concern with respect to an increased risk of adverse progeny outcome. Paternal counseling, after a chemical exposure or treatment with drugs that are known to be toxic to male germ cells, would be greatly facilitated by the development of a battery of new tests to detect the effects of exposure on sperm chromatin and function.

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## Can we protect spermatogenesis against testicular insults?

### *Male reproductive toxicology*

M.L. Meistrich

#### Exposures to testicular insults

The germinal epithelium of the human testis is often subjected to chemical and physical insults as a result of medical, occupational, and environmental exposures. Among necessary medical treatments, anticancer agents and immunosuppressive drugs, particularly radiation and alkylating drugs, frequently cause temporary or permanent reductions in sperm count (oligospermia), and may even result in complete lack of sperm (azoospermia). Industrial solvents, such as bromopropane, and compounds released from plastics used in food containers and medical tubing, such as phthalates and bisphenol A, also damage spermatogenic function. Anabolic steroids, hormone antagonists, and environmental compounds such as bisphenol A or the fungicide vinclozolin can disrupt hormone signals essential for spermatogenesis (Chapters 28,29). Methods to prevent these damaging effects and to restore gonadal function after the toxic treatment are of great importance to men who desire to have children.

#### Nature of testicular insults

Some examples of the chemical, cellular, or endocrine nature of toxic insults are given below.

Radiation and alkylating agents, including those used in cancer chemotherapy, and such environmental toxicants as dibromochloropropane, induce free radicals, reactive oxygen species, and reactive alkyl groups that directly damage the DNA. These reactive intermediates can be scavenged and chemically detoxified, which prevents them from even producing the damage to DNA.

The anticancer agents preferentially kill proliferating cells, which in the testis are specific germ cells, primarily the rapidly proliferating differentiating spermatogonia, but the more slowly proliferating stem spermatogonia are somewhat susceptible (Fig. 1). As the surviving cells progress through spermatogenesis with fixed kinetics, the gap left by killing of spermatogonia will result in temporary depletion of later germ cells and reductions in sperm counts. But recovery should occur from the surviving stem cells within several months.

Killing of the stem cells reduces the potential for recovery and results in prolonged or permanent azoospermia. In addition, the somatic cells of the testis, which in adults are generally nonproliferating, survive cytotoxic exposures but suffer functional damage that may hinder the recovery of spermatogenesis from the surviving stem spermatogonia. One of these somatic cells, the Sertoli cell, is essential for the structural organization, nourishment, and regulation of the germ cells and is a direct target of some toxicants (phthalates). Damage to these somatic cells often leads to apoptotic death of the germ cells.

Endocrine disrupting chemicals can, by several different mechanisms, reduce the production or action of testosterone, which is essential in high concentrations in the testis for spermatogenesis to proceed (Chapter 8). Anabolic steroid analogs can inhibit testosterone production in the Leydig cells, 5-alpha reductase inhibitors can reduce testosterone activation, and vinclozolin and other antiandrogens can block testosterone action. These agents can affect fetal and pubertal testicular development and have irreversible effects on sperm output in later adulthood.

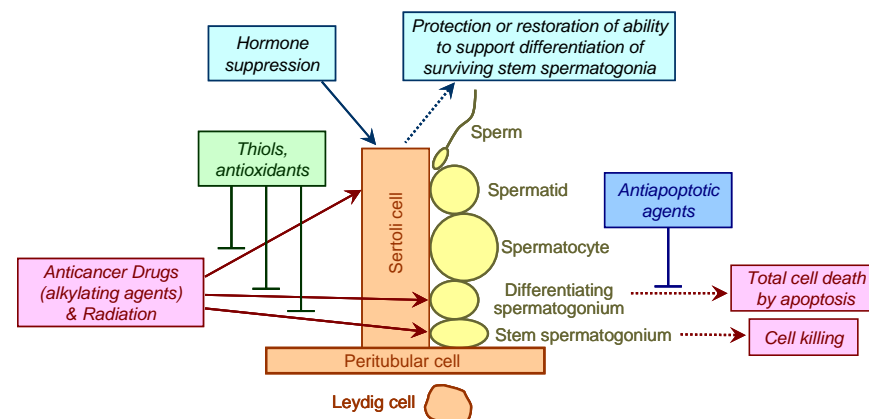


FIG. 1. Diagrammatic representation of action of anticancer agents on specific cells of the testis and outcomes. Different targets for protection are shown: blocking the initial damage (thiols, antioxidants), restoring somatic cell function to enable surviving spermatogonia to reinitiate spermatogenesis (hormone suppression), and enabling the damaged cells to survive by blocking apoptosis (anti-apoptotic).

#### Various protective approaches

Methods to prevent these damaging effects and to restore gonadal function after the toxic treatment have been tested by a variety of physical, biochemical, and biological approaches in experimental animals. The only

example of protection from testicular injury that has been reliably used in humans is shielding of the testes during radiation therapy. The testes can be well-shielded from the direct radiation beam, although scattered radiation still presents some concerns.

Among the methods that have been limited to experimental animals are free radical scavengers, antioxidants, and reducing compounds, including thiols; these can protect against the primary damage (Fig. 1). Amifostine, originally designed to selectively protect cells against free radicals produced by radiation, protects some normal tissues but not tumors against chemotherapy. Although it does partially protect mouse spermatogenesis against radiation, amifostine shows direct toxicity to stem spermatogonia, which in conventional fractionated anticancer regimens would outweigh the protective benefit.

Reducing blood flow to testes by transient ligation or cryptorchidism has been investigated to protect against damage by chemotherapy drugs with short half-lives. Indeed reversible ligation does protect animal testes against adriamycin and produced minimal direct damage, but its potential to cause ischemic damage remains a concern.

Anti-apoptotic compounds, such as sphingosine-1-phosphate, have been used against toxicants that kill germ cells by apoptosis. However, this does not offer significant protection of the testes.

Other empirical approaches, including administration of prostaglandins, vitamins, growth factors, and cytokines, have been tested in animals, and have demonstrated some protection against toxic effects. However, there is no evidence of therapeutic benefit against anticancer agents, i.e., that these treatments would provide more protection of the testis than of the tumors themselves.

### **Hormonal approaches to protection**

The concept of hormone suppression for protecting gonadal function from cytotoxic exposure was based on the greater resistance of non-cycling cells than rapidly proliferating cells to killing by antineoplastic agents. It was erroneously thought that suppression of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone levels would reduce the proliferation of the target cells and render them more resistant to the toxicant. But suppression of these hormones only blocks the completion of spermatogenesis and has no effect on the proliferation of the crucial target cells, the stem spermatogonia.

Despite this incorrect theoretical basis, hormonal suppression of rats prior to, during, and even after exposure to chemotherapy or radiation dra-

matically enhances the subsequent recovery of spermatogenesis. However, this is a result of protection or restoration of the function of the somatic cells of the testis to support the differentiation of the surviving stem spermatogonia and not protection of these spermatogonia from being killed. Hormonal suppression can modestly stimulate recovery of spermatogenesis from cytotoxic insults in mice but not in non-human primates.

Clinical trials of protection or restimulation of spermatogenesis by hormonal suppression have generally been unsuccessful, with only one study showing protection. We will have to know more about the mechanisms underlying the protection and recovery of spermatogenesis by hormonal suppression in rodent models, and their interspecies similarities and differences, before we can more rationally apply the results to human spermatogenesis.

### **Conclusions**

As yet there are no proven methods for protecting the human germinal epithelium when it is directly exposed to chemical or physical insults nor proven therapeutic options to improve spermatogenesis after damage has occurred. However, protection may be achieved by removal of germ cells prior to exposure and storage for later use. Sperm banking done prior to medical exposures routinely results in successful pregnancies. In experimental animals, cryopreservation of spermatogonial stem cells or testicular tissue, and later reintroduction into the testicular tubules or transplantation into a subcutaneous site, respectively, result in production of viable sperm that yield live offspring. Awareness of such novel research in experimental animals designed to either protect the germinal epithelium from toxic insults or restore its function after the insult could lead to their application to humans.

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## What male contraceptives currently exist and what is the outlook for new male contraceptives?

J.K. Amory and W.J. Bremner

### Vasectomy and condoms

Efficacious contraceptive options currently available to men are vasectomy and condoms, which together account for 20-30% of all current contraception in the US. Vasectomy is a safe, simple, outpatient surgery performed under local anesthesia in which the vas deferens is severed and the ends ligated and/or cauterized through a small scrotal incision. Vasectomies are highly effective with a failure rate of less than 1%. The “no scalpel” technique, in which a single puncture is made midline in the scrotal raphe with scissors, is probably superior to older techniques. Drawbacks to vasectomy include a delay in the onset of azoospermia of several months, pain and rare infections. While post-operative pain resolves quickly, some men will experience chronic scrotal discomfort. Vasectomy is most appropriate for men who no longer wish to father children, since surgical reversal does not always restore fertility.

Condoms made of animal intestine have been used as a means of male fertility control for several hundred years. Since 1920, most condoms have been made of latex rubber, which affords some protection against sexually transmitted diseases including HIV/AIDS. However, condoms have a marginal contraceptive efficacy with pregnancy rates approaching 10-15% per year. This is mainly due to improper or inconsistent usage, although condom breakage can occur. In addition, latex allergies can be a problem for some users. For these men, polyurethane condoms are a reasonable alternative.

### Experimental male contraceptives

Efforts are ongoing to develop new methods of contraception for men. The approach that has been tested most extensively involves the administration of testosterone, which functions as a contraceptive by suppressing the secretion of luteinizing hormone and follicle-stimulating hormone from the pituitary (Chapter 8; Fig. 1). This deprives the testes of the signals required for spermatogenesis and results in markedly decreased spermatogenesis and effective contraception in most men. Male hormonal contraception is well tolerated, fully reversible, and appears to be free from serious adverse effects. Unfortunately, regimens in which testosterone is

used alone fail to completely suppress spermatogenesis in some men, meaning that the potential for fertility persists. Because of this, combinations of testosterone and progestins, which synergistically suppress gonadotropins, have been studied (see below).

Normally, sperm concentrations exceed 15 million/ml. The absence of sperm in the ejaculate, a condition called azoospermia, makes fertilization impossible. A sperm concentration below 1 million/ml., or “severe oligospermia,” is associated with a pregnancy risk of approximately 1% per year. Therefore, achieving severe oligospermia in all men is considered a reasonable goal of male contraceptive development. Because male hormonal contraceptives inhibit sperm production, 2-3 months elapse until the sperm concentration in most men is fully suppressed. In addition, for unknown reasons Asian men exhibit higher rates of azoospermia than non-Asian men.

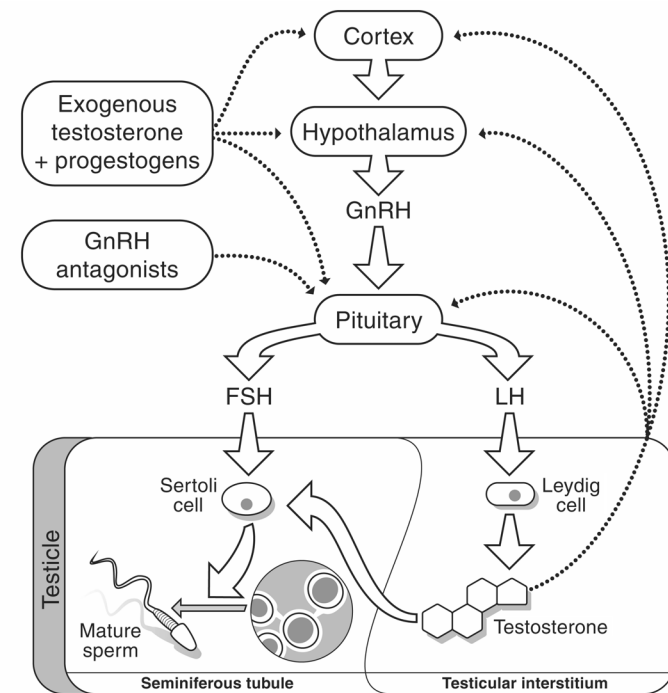


FIG. 1. Spermatogenesis and male hormonal contraception. Solid arrows, promotes spermatogenesis; dashed arrows, inhibits spermatogenesis. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone. Negative feedback of testosterone occurs at the level of the pituitary, the hypothalamus and the cortex.

The World Health Organization conducted two large, multicenter trials of injections of testosterone enanthate for male contraception. The first enrolled 271 subjects who were administered 200 mg testosterone enanthate by intramuscular injection weekly for six months. Sixty percent of the men in this study became azoospermic, and an additional 30% became severely oligospermic. The fertility of 119 of the azoospermic men was then tested in a 12-month efficacy phase. In these couples, only 1 pregnancy occurred, corresponding to a pregnancy rate of less than 1%.

The second study examined the fertility of men who became either azoospermic or oligospermic (variably defined in this study as less than 3-5 million sperm/ml. ejaculate) with injections of 200 mg of testosterone enanthate weekly. Of a total of 399 mostly Asian men, all but 8 (2%) became oligospermic or azoospermic. There were no pregnancies fathered by the men who became azoospermic, and fertility was reduced to 8.1 pregnancies per 100-person years in the men who suppressed to less than 3 million sperm/ml. Therefore, the overall failure rate was 3.4%, for an overall contraceptive efficacy of 96.6%. All subjects returned to normal after the testosterone injections were discontinued. These two studies demonstrated that testosterone is safe, reversible and highly effective in a majority of men. However, they also demonstrate that pregnancy is possible even at very low sperm concentrations.

Side effects in these trials included a 10-20% decrease in serum HDL-cholesterol, occasional acne and small, reversible reductions in testicular volume. Importantly, cognitive function, well-being, quality of life and sexual function were not adversely affected. The regimen was found to be better than expected by a majority of subjects; however, the requirement for weekly intramuscular injections led twelve percent of the subjects to discontinue participation.

As a result, recent research in male hormonal contraception has focused on formulations of testosterone that can be administered less frequently, such as testosterone undecanoate. Monthly injections of 500 mg of testosterone undecanoate were studied in 308 Chinese men, 299 of whom suppressed to sperm concentrations below 3 million sperm/ml. Two hundred and ninety six of these men went on to use the testosterone undecanoate injections as a sole means of contraception for one year. In these couples, only one pregnancy occurred, for an overall contraceptive efficacy of 97%. Testosterone undecanoate has also been combined with the progestin etonogestrel in an implant formulation in 350 subjects with greater than 90% suppression of spermatogenesis to less than 1 million sperm/ml.

Why some men fail to fully suppress their spermatogenesis despite profound suppression of gonadotropins is unknown. Since there are few

significant differences in the gonadotropin levels during treatment among men who suppress to azoospermia and those who do not, the degree of gonadotropin suppression itself is not the answer. Therefore, genetic, dietary or other factors likely play a role. Clearly, further studies of the control of spermatogenesis are needed.

## Summary

Vasectomy and condoms are widely used, effective forms of male contraception. Experimental testosterone-progestin based male hormonal contraceptives reversibly suppress human spermatogenesis without severe side effects in most men; however, a uniformly effective regimen has remained elusive. Nevertheless, it is possible that improvements in this approach may soon result in the clinical introduction of a safe, reversible and effective form of male contraception.

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## What are the consequences of sexually transmitted infections on male reproduction?

F.R. Ochsendorf

Sexually transmitted infections/diseases (STI) are caused by organisms sharing the following properties: 1) very sensitive to physical/chemical factors, hence only transmittable by direct contact, 2) only infectious for humans, 3) colonizing the genital region, 4) often causing only mild symptoms and 5) not leading to immunity. As a consequence concurrent infections with different STD-agents may occur at the same time (Table 1).

### Mechanism of damage

Fertility may be impaired by organ damage due to the organism itself, cell damage via mediators of inflammation, inflammatory obstruction of the excurrent ductal system, or binding to spermatozoa. Thus, with respect to the impact on male reproductive system, chronic or inadequately treated STI's are more relevant than the acute infections.

### Relevance

The difference in the prevalence of STI's in different regions of the world as well as the access to their adequate diagnosis and treatment explain conflicting data on the relevance of these infections in male infertility. In western countries STI's only account for a minority of inflammatory damage in the reproductive tract. However, In other parts of the world STI's can severely impair male fertility: men with a history of penile discharge, painful micturition and genital ulcers, who did not seek adequate treatment for these symptoms, are more likely to be infertile than men without these symptoms or are adequately treated. Recently it was shown that 18.7% of semen samples (45/ 241) of asymptomatic men seeking infertility investigation contained DNA from STI-pathogens (e.g., CMV, HPV, HSV, HHV6, EBV, *C. trachomatis*). There was no difference in prevalence between samples with or without leukocytospermia. The presence of STI-DNA in semen samples was found to be associated with impairment in semen parameters (e.g., concentration, motile sperm concentration, total sperm count and neutral alpha-glucosidase concentration). However it has to be kept in mind that the presence of a germ does not necessarily mean "infection", as the pathogen may just colonize the tissue without causing a clinical infection with pathological sequelae. This fact may also explain some conflicting data in the literature. Another impact of STI's is their potential to increase the rate of

HIV-transmission. Thus, adequate diagnosis and treatment of STI's is not only relevant for the prevention of long-term negative consequences for fertility but also for the prevention of HIV-spreading.

### Relevant infections

Various common pathogens that can be isolated in the male reproductive tract are listed in Table1. Currently, literature supports the association of a significant negative impact on male fertility with infection of gonococci and HIV, while the roles of other pathogens such as *chlamydia trachomatis*, *ureaplasma urealyticum*, HSV, HPV and *trichomonas vaginalis* in impairing male fertility remain controversial.

#### Bacteria and protozoa

A review of the literature could not demonstrate a general link between urethritis and male infertility. Gonorrhoea, however, can cause urethral strictures as well as a strong impairment of testicular functions. In female, *C. trachomatis* infection can impair fertility by tubal obstruction. On the other hand, its impact on male fertility remains to be established although some epidemiological data points to the risk of subfertility in men (and women) with a past *C. trachomatis* infection. The exact role of Mycoplasmae, i.e. *M. hominis*, *Ureaplasma urealyticum* and *M. genitalium* has still to be elucidated. *U. urealyticum* may cause infertility via deleterious effects on sperm chromatin and DNA integrity, leading to impairment of embryo development. *Trichomonas vaginalis* was more often found in infertile men than in fertile controls; however, no effect on motility or sperm-mucus interaction has been demonstrated (Soper 2004).

#### Viruses

At present the clinical relevance of viruses as a cause of male infertility is largely unknown. Cytomegalovirus (CMV) and human herpes virus type 6 (HHV-6) were demonstrated in semen without an association with impaired semen parameters. The data on the relevance of herpes simplex virus (HSV) as well as human papilloma virus (HPV) and their associations with sperm parameters are conflicting (Ochsendorf 2006).

*HIV.* Infected leukocytes are the most relevant source of human-immunodeficiency virus (HIV) in the male reproductive tract. In AIDS patients oligozoospermia, azoospermia, orchitis and hypogonadism were reported. Spermatozoa morphology is particularly impaired with progression of the disease. Disturbed function of the seminal vesicles and prostate gland may explain the decrease in semen volume and increase in ejaculate viscosity. With the latest advances in drug-therapies, HIV infection is considered a chronic disease. The safety of both the uninfected partner and the potential



offspring are of particular concern. In a sero-discordant couple the risk of acquiring HIV is dependent on the viral load in semen. Depending on the semen quality, spermatozoa can be used for intrauterine insemination, IVF or ICSI after adequate preparation of the ejaculate. The semen has to be washed free of HIV by a gradient technique and the success of this procedure has to be controlled prior to use. A thorough counselling of both partners on the therapeutic options, psychosocial and economic aspects with respect to their sexual health and reproductive health is thus necessary.

**Table 1: Sexually transmitted infections: pathogens and relevance for male fertility; + = clinical relevance demonstrated; ? = relevance possible but not yet proven**

Disease	Pathogen	Relevance for infertility
<b>Bacteria</b>		
Gonorrhoea	Neisseria gonorrhoea	Male + Female +
Chlamydia infection	Chlamydia trachomatis (D-K)	Male ? Female +
Urethritis (due to)	Ureaplasma urealyticum	May impair motility, sperm chromatin ?
Syphilis	Treponema pallidum	Co-factor for transmission of HIV Gummatous testicular lesions: + Female +
Chancroid	Haemophilus ducrey	-
Lymphogranuloma venereum	Chlamydia trachomatis (L1-L3)	-
Granuloma inguinale	Calymmatobacterium granulomatis	-
<b>Viruses</b>		
AIDS	HIV	Male +
Mononucleosis	CMV	-
Asymptomatic infection	HSV	Impaired semen quality ?
Asymptomatic infection	HPV	Impaired semen quality ?? Asthenozoospermia
Asymptomatic infection	Adenovirus	Infertility ?
Mumps	Mumps virus	Testicular atrophy +
<b>Protozoa</b>		
Urethritis (Prostatis, Epididymitis) due to	Trichomonas vaginalis	? controversial
<b>Yeasts</b>		
Balanitis, Urethritis (due to)	Candida albicans	-

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## What types of testicular cancers occur in men? What is their prognosis? How effective are existing therapeutic approaches?

N.E. Skakkebaek

### Germ cell tumors

Testicular cancer has been increasing in incidence all over the world and is now the most common malignancy among young men. The vast majority of these tumors are germ cell tumors. They are believed to originate from primordial germ cells or gonocytes, which fail to differentiate to spermatogonia in fetal life. However, these precursor cells, which persist during childhood do not develop into invasive cancer until after puberty. The peak incidence occurs around age 20-40 and these tumors are very rare in old men. Germ cell neoplasia can be non-invasive for many years as a preinvasive *carcinoma in situ (CIS)* pattern, where the normal germ cells of the seminiferous epithelium are replaced by a row of carcinoma in situ germ cells (Fig 1).

Such tubules may occupy from a few to almost 100 percent of the seminiferous tubules. In these tubules with CIS, Sertoli cells are always present although there are rarely any normal germ cells. Recent research has shown that CIS cells express stem cell markers, e.g. Oct-4, NANOG, AP-2 gamma and C-Kit. It is thought that patients with untreated CIS will eventually develop invasive tumors, either seminomas or non-seminomas. It should be noted that seminomas are in fact gonocytomas (not “semen” tumors, as the name may suggest). Four subtypes of non-seminomas exist, namely embryonal carcinomas, terato-carcinomas, choriocarcinomas and yolk sac tumors. In many cases elements of seminomas and non-seminomas may co-exist in the same testis.

The main reason for dividing the germ cell tumors into these categories is that prognosis and treatment for seminomas and non-seminomas are different. Prior to the introduction of cis-platinum based chemotherapy that was introduced in the 1980s, seminomas had a rather good prognosis, while non-seminomas were associated with a higher mortality rate, particularly if they had metastasised at the time of the initial presentation.

### Diagnosis

The diagnosis of a testicular tumor must be based on taking a careful history. In most cases the patients present with a testicular mass with minimal

discomfort. Previous history of cryptorchidism may be elicited. Physical examination on careful palpation of both testes generally reveals a hard nodule. Further clinical evaluation may include testicular sonogram and measurement of serum tumor markers, including human chorionic gonadotropin (elevated in cases of choriocarcinoma) and alpha-foetoprotein (elevated in yolk-sac tumor). Unfortunately the diagnosis of testis cancer may be delayed due to the patient's unawareness of the signs of tumor or the doctor's misclassification of the tumor as an infection of the epididymis.

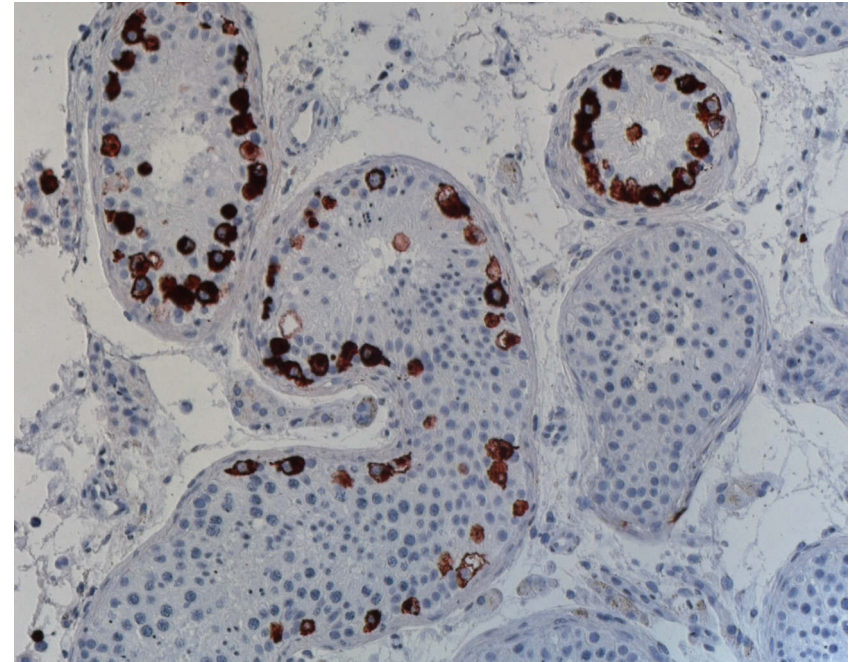


FIG. 1. Testicular biopsy showing several tubules with carcinoma in situ cells (the brown-red stained cells along the basal membrane). Placental like alkaline phosphatase (PLAP) immunostaining. The two upper tubules contain CIS cells and Sertoli cells and no normal germ cells, while the lower CIS tubule also contain normal germ cells. Note - for comparison - the tubule to the lower right with normal spermatogenesis.

### Treatment and prognosis

The initial management of testicular germ cell tumors includes radical orchiectomy and tumor staging using computer-tomogram (CT) to evaluate for metastasis to the mediastinum of the chest and retroperitoneum.

In the absence of signs of metastases and depending on the histology of the testis tumor and the levels of serum tumor markers, it is adequate for the patients to have frequent follow-up visits and regular imaging procedures. In cases with more aggressive types of tumor, as suggested by the histological subtypes, elevated tumor markers or the presence of metastasis, combination chemotherapy and/or radiation therapy may be needed. Additional surgery to remove and evaluate the metastatic tumor tissues may be needed to evaluate its nature, as it can be different from the original tumor in the testis. Pure seminomatous metastases are generally very sensitive to radiation therapy. In cases where both seminomas and non-seminomatous tumor tissues are present in the same patient, the treatment strategy is directed towards the non-seminomatous tissue, that require a more aggressive form of therapy such as cis-platinum based combination chemotherapy. With the advances in diagnosis and treatment option, the prognosis of testis cancer has improved dramatically in the past decade with a five-year survival rate of 90-95% with a good quality of life.

Most cases of testicular germ cell cancer are unilateral, but the contralateral testis carries an increased risk of developing a second germ cell tumor, which may present months or years after the initial presentation. The increased risks of testis cancer in the contralateral testis is not due to metastasis from the original tumor, but to the presence of carcinoma in situ, found in 5% of the contralateral testis at the time of initial diagnosis. Therefore some oncologists advocate routine biopsy of the contralateral testis at the time of the primary orchiectomy. If the neoplasia is detected at this preinvasive stage, adjuvant treatment with 16-20 Gy of radiation therapy to the contralateral testis may avoid the need of a second orchiectomy in future and preserve some testicular function for the patient.

### Risk factors

Generally, men with testicular germ cell cancer are less fertile than men in the general population, even before they develop their tumors. Testis cancer is also associated with a history of undescended testis and hypospadias. The concept of testicular dysgenesis syndrome ties together many of the related conditions, with germ cell cancer being the more severe one. Besides CIS lesions, dysgenetic testes may contain Sertoli-cell-only tubules, undifferentiated tubules, Leydig cell clumps and microliths. Because of the risks of infertility, we recommend that patients with a testicular tumor should be advised on sperm cryopreservation to preserve their fertility, particularly prior aggressive cancer treatment (Chapter 14).

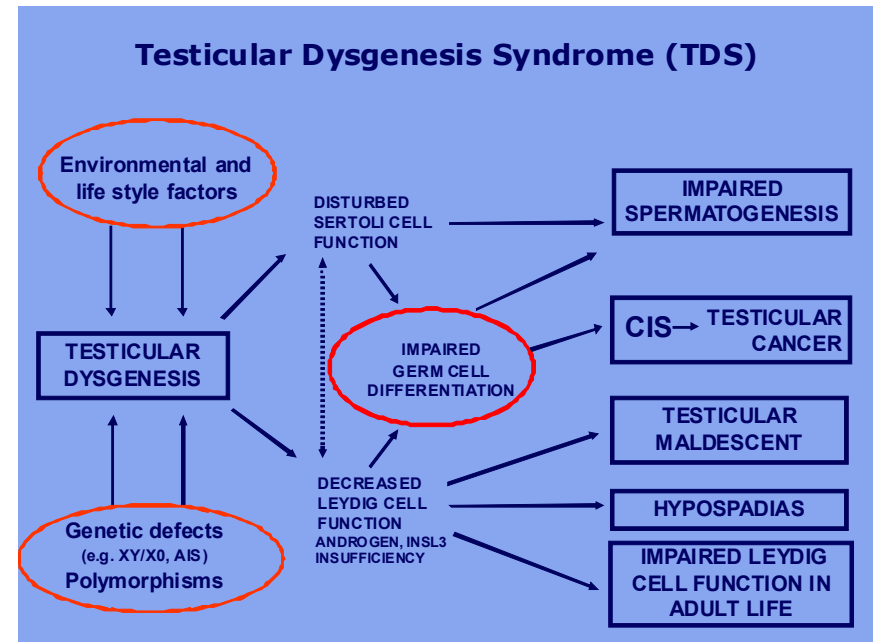


FIG. 2. Pathogenesis of testicular dysgenesis syndrome. Note that both genetic and environmental factors may contribute to testicular dysgenesis(modified from(9))

### Other tumors of the testis

Rarely, germ cell tumors may occur in neonates and infants. Such tumors have different origins and pathogenesis than the germ cell tumors described above and are more frequently benign than those seen in young adults. Men above age 60 may harbour a special germ cell tumor called spermatocytic seminoma. In contrast to classical seminoma which occurs in younger men, spermatocytic seminoma have completely different pathogenesis. They are not biologically linked in any way. Spermatocytic seminoma is derived from spermatogenic cells, rather than from gonocytes as in the classical seminoma. Furthermore, the natural history of classical seminoma is more aggressive with higher metastatic risks than spermatocytic seminoma.

Somatic cells of the testis, including Sertoli cells and Leydig cells very rarely develop tumors, with most being benign in nature. Leydig cell tumor may often result in elevated production of testosterone which may be

converted into estrogens through peripheral aromatization and thereby causing gynecomastia. Finally lymphoma and leukemias may infiltrate the testes and falsely simulate a gonadal tumor. Thus clinician managing testis tumor should be familiar with the various clinical presentations.

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## **How do erections occur? How common is erectile dysfunction, what is its etiology, and how do you evaluate men with this problem?**

P.M. Pierorazio and A.L. Burnett

### **How do erections occur?**

Penile erection requires elaborate orchestration of neural, vascular and hormonal processes in the proper psychological setting. The function occurs with the filling of the corpora cavernosa, spongy vascular chambers of the penis, with blood to create a rigid organ usable for sexual intercourse. Erections are initiated and maintained by two main processes: relaxation of the arteries of the corpora cavernosa to allow increased inflow of blood, and increased resistance of outflow venous channels to maintain tumescence. Increased resistance to outflow is a passive process – the venous channels are compressed against the tunica albuginea by the expanding penile tissue. The increased inflow is a complicated neurohormonal process, whereby messenger molecules (nitric oxide, cGMP, cAMP, and others) released by local neurons lead to smooth muscle relaxation, arterial dilation and increased blood flow.

### **How common is erectile dysfunction?**

Erectile Dysfunction (ED) is defined as “the inability to attain and/or maintain penile erection sufficient for satisfactory sexual performance.” (AUA Guidelines, 2005) The prevalence of ED increases with age. For men less than 40 years old, the worldwide rate of ED ranges from one to nine percent. For men in their sixties, rates vary from 20 to 40%. As much as 50 to 75% of men in their 70’s and 80’s will have ED. In the US, it is estimated that over 600,000 men each year will develop ED. The risk of ED increases with diabetes mellitus, heart disease, and hypertension.

### **What is its etiology?**

Erectile dysfunction can manifest in many ways and have many physiologic causes. Patients may be unable to achieve a complete erection, or to maintain the erection; they may have pain with erections or changes in erogenous penile sensation. These problems may be caused by dysfunctional neurons that carry messages to the penis, or there may be an

inability of the blood vessels to deliver adequate blood flow to create an erection.

The causes of ED form five specific categories: (1) vasculogenic, due to either arterial or venous problems, (2) neurogenic, (3) endocrinologic, (4) related to medications or (5) psychogenic. The most common etiology of ED is by far vasculogenic, with venogenic (cavernosal) causes believed to be slightly more common than arteriogenic causes. Vasculogenic etiologies can be related to cardiovascular disease, like atherosclerosis, that leads to arterial insufficiency or degenerative changes of the fibroelastic tissue of the penis. Neurogenic causes, that are estimated to make up 10 to 19% of ED, may be related to neurological disease (like multiple sclerosis) or result from physical damage to nerves either during pelvic surgery or from traumatic injuries to the pelvis. Endocrinologic causes of ED are rarer; however, thyroid hormone or sexual hormone abnormalities are more common in patients with ED than they are in the general population. Many cases of ED are medication-related. The most common culprits are anti-hypertensive medications, especially beta-blockers, that lead to decreased blood perfusion to the penis for erection to occur. Other medications, including many classes of psychiatric medications and medications that reduce male sexual hormones (i.e. testosterone) are related to ED. Psychogenic causes are believed to play a role in up 90% of ED (often combined with another etiology). The most common psychogenic causes are depression, anxiety, and stress, although many psychiatric disorders can contribute to ED. Many disease states that are related to ED can have more than one categorical etiology. For example, diabetes mellitus can contribute to both vasculogenic and neurogenic dysfunction that can hinder the ability to achieve erection.

### **How do you evaluate men with this problem?**

In addition to eliciting details regarding the etiology of ED, it is important to understand if the ED is a problem of physical function (erections do not occur or are unsatisfactory) versus performance (erections can be achieved but not during sexual stimulation). The functional types can be thought of as organic ED versus psychogenic ED; organic ED refers to a physical aberration that prevents the achievement of a functional erection, and is usually vasculogenic, neurogenic, endocrinologic in nature, or medication-related. In the absence of these conditions, psychogenic ED may be inferred. Etiologic and functional classifications of ED are demonstrated in the Fig. 1.

Therefore, the most important measures to evaluate ED are a good history and physical examination. First, understanding the circumstances of the patient's ED can help delineate the type of ED and in some cases the

etiology of ED. Important details of the sexual history specifically regarding erection include the degree of rigidity achieved, the presence of nocturnal or early-morning erections, the ability to have sexual intercourse or masturbate and the presence of any deformities or curvatures of the penis. Questionnaires like the Sexual Health Inventory for Men (SHIM), also known as the International Index of Erectile Function, 5-item version (IIEF-5), are standardized tools used by physicians to understand and rate the severity of each patient's ED. In general, high scores indicate normal erectile function while lower scores indicate worsening degrees of ED.

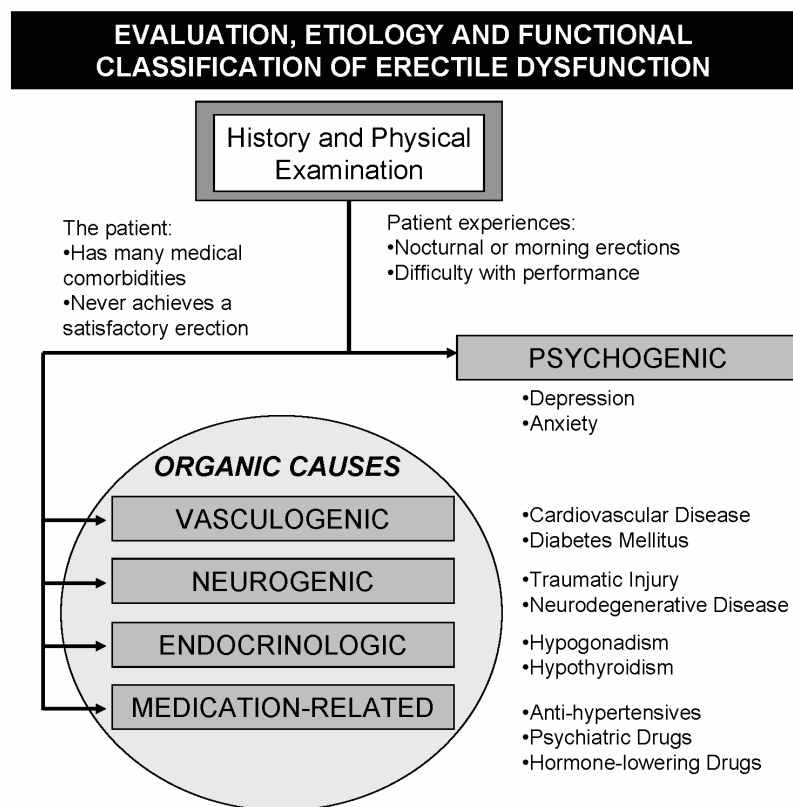


FIG. 1. Evaluation, Etiology and Functional Classification of Erectile Dysfunction (ED). Common examples of disease states in each category are listed adjacent to each.

Many of these details can help the evaluating physician discern between organic and psychogenic types of ED. Once it is clear (or highly suspicious)

that an organic type is responsible for ED, it is important to ascertain the patient's medical comorbidities, medical and surgical history, and medications. By capturing these details, physicians may find correctable causes of ED. For example, a patient whose ED coincides with starting metoprolol (a common anti-hypertensive medication) may warrant a trial of new medication to control his blood pressure. Additionally, it is extremely important to assess each patient's risk factors for cardiovascular disease. These risk factors include older age, hypertension, hyperlipidemia, diabetes mellitus, obesity, cigarette smoking and a sedentary lifestyle. By determining the presence of these factors, which are potentially modifiable, patients can make changes that can improve their erections and decrease their risk of having a cardiovascular event (heart attack or stroke) in the future. Interestingly, recent data have demonstrated that ED may be an early sign and risk factor for cardiovascular disease.

Physical examination should involve a thorough inspection of the genitalia for deformities that may contribute to ED. Peyronie's Disease for example is a curvature of the penis that is associated with ED. A small or non-present testis or testes may be indicators of hypogonadism. In addition, a thorough vascular and neurological examination including peripheral pulses and reflexes may suggest vasculogenic or neurogenic causes of ED.

Once a thorough history and physical examination are completed there are many laboratory and physiological tests that can be performed to measure the degree of ED and delineate certain causes of ED. Serum lipid levels and hormone levels can identify many cardiovascular and endocrinologic etiologies of ED. Ultrasound and vascular imaging technologies can identify whether or not there is adequate blood flow to the penis. In general, many evaluations of ED are complete without the need for complicated testing – a thorough history and physical examination are often sufficient before initiating ED treatment.

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## How to treat erectile dysfunction

M.C. Raynor and W.J.G. Hellstrom

### Introduction

Management of erectile dysfunction (ED) may vary considerably depending on the etiology. Following a focused evaluation of the ED patient (Chapter 34), therapy can usually be recommended based on the underlying mechanism. In most men, a thorough history, physical exam, and basic laboratory studies should suffice. Further evaluation with more invasive studies may be indicated for specific patient populations (Peyronie's disease, pelvic injury, endocrine disorders, young males unresponsive to oral agents, etc.).

### Non-pharmacologic management

Non-pharmacologic management of ED can be used with success in some men. Lifestyle modifications may be implemented to alter certain risk factors for ED. Obesity and low levels of physical activity are recognized to be significantly associated with ED. Men with higher body mass index (BMI) and a sedentary lifestyle were at most risk for ED. In addition, cigarette use increased the risk of ED in men with other risk factors, such as hypertension or diabetes mellitus. One randomized trial demonstrated that men who lost weight and increased physical activity had significant improvements in erectile function.

Medications can also have a profound effect on sexual function. These effects can range from decreased blood pressure, hormonal alterations, decreased sexual arousal, or central suppressive effects (Chapter 34). Treatment of hypertension is one of the most common causes of medication-induced ED. Non-specific  $\alpha$ -adrenergic antagonists and thiazide diuretics are commonly associated with ED. Spironolactone interferes with testosterone synthesis and antidepressants can have a significant impact on sexual function. Monoamine oxidase inhibitors (MAOIs) and selective serotonin reuptake inhibitors operate through central mechanisms and can decrease arousal. Simply altering medications to a different class may be successful. Calcium channel blockers and angiotensin converting enzyme (ACE) inhibitors both have decreased effects on sexual function. Other antidepressant medications, such as bupropion or venlafaxine, may also have decreased inhibitory effects on sexual function.

### Psychosexual counseling

Psychological counseling has been used with success in various groups of men with ED. In general, men with psychogenic ED are the main beneficiaries of counseling. These men generally are found to be physiologically normal in terms of erectile function but may suffer some cognitive impairment that affects sexual function. For example, young men with congenital penile chordee or infertility may develop psychogenic ED secondary to dysmorphia or anxiety. Individual and couples counseling can be successful in improving self-confidence and decreasing anxiety surrounding sexual function or body image. Counseling can also be utilized in conjunction with pharmacologic strategies to improve outcomes.

### Vacuum erection device

One non-pharmacologic management strategy that aims to physically produce an erection, as opposed to modifying risk factors, is the vacuum erection device (VED). The VED is a vacuum cylinder with a constriction ring that is placed at the base of the penis once the penis is engorged. Advantages of the device include its ability to produce a rigid erection sufficient for intercourse, including engorgement of the glans. Although relatively inexpensive, it can also cause pain at the band site, decreased ejaculate volume or anejaculation, and bruising.

### Pharmacologic management for ED

#### *Phosphodiesterase type-5 inhibitors*

Pharmacologic management has become the mainstay of treatment for most men with ED following the advent of phosphodiesterase type-5 (PDE-5) inhibitors. Approved in 1998, sildenafil (Viagra, Pfizer, NY, NY) significantly changed the management of ED for most men. Later, vardenafil (Levitra; Bayer, GlaxoSmithKline, Schering-Plough) and tadalafil (Cialis; Lilly) were approved for use. All three medications have similar efficacy and side effect profiles. These medications work by potentiating the effect of nitric oxide on cavernosal smooth muscle. Nitric oxide stimulates guanylyl cyclase and leads to elevated cGMP levels. These second messengers then decrease intracellular calcium levels, which leads to smooth muscle relaxation and penile erection. PDE-5 breaks down cGMP to GMP, thus, inhibition of this enzyme increases cGMP levels (Fig. 1).



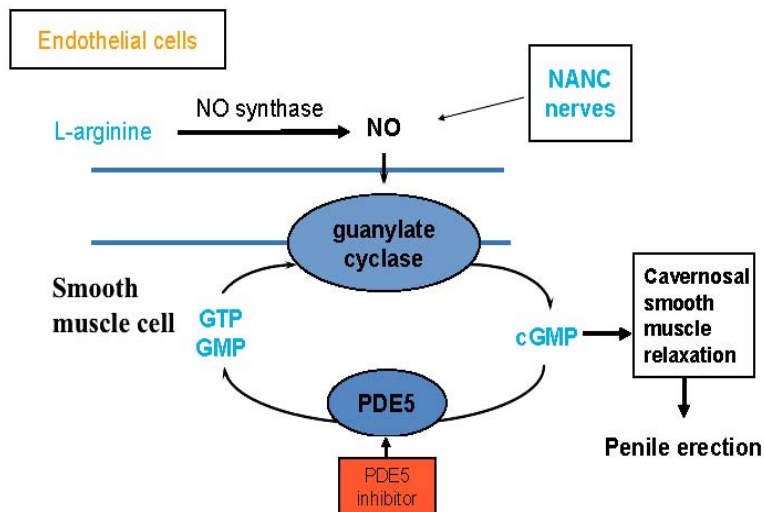


FIG. 1. The role of nitric oxide and cGMP in the physiology of erection.

Side effects include flushing, headache, muscle ache, and visual disturbances. These effects are likely due to cross-reactivity with other phosphodiesterases (usually PDE-6 and 11). There have been isolated reports of vision loss following use of PDE-5 inhibitors, known as non-arteritic anterior ischemic optic neuropathy (NAION). Men with retinal conditions including retinitis pigmentosa should not use these medications. Vardenafil carries an added warning about cardiac conduction defects as it may have an effect on the QT interval, therefore, some men taking anti-arrhythmics should avoid vardenafil. PDE-5 inhibitors are also contraindicated in men taking nitrates because of the risk of an unsafe drop in blood pressure. Men taking  $\alpha$ -adrenergic antagonists for BPH should be cautioned regarding the risk of decreased blood pressure with concomitant use. Patients undergoing radical prostatectomy for prostate cancer and men with diabetes mellitus all have shown improvements in erectile function with use. Unless a contraindication for use exists, PDE-5 inhibitors have become first-line therapy in the treatment of ED.

#### Intracavernosal therapies

If PDE-5 inhibitors fail after proper instruction has been given or if a contraindication for use exists, men may consider alternative medical treatments. Intracavernosal injection (ICI) agents have been around for over 25 years. These vasoactive agents are injected directly into the cavernosal tissue and

produce an erection. Medications include papaverine, phentolamine, and alprostadil. Papaverine is a non-selective PDE inhibitor that increases intracellular cAMP and cGMP levels. Phentolamine is an  $\alpha$ -adrenergic antagonist that increases presynaptic norepinephrine levels. Alprostadil works by increasing intracellular cyclic AMP levels and decreasing intracellular calcium. These medications are used alone or in combination for injection. Side effects include painful erection, priapism, and increased incidence of cavernosal fibrosis (mainly with papaverine and phentolamine). Men taking anticoagulants should be advised to apply manual pressure for several minutes following injection to avoid hematoma formation. Alprostadil is also available in a urethral suppository form (MUSE, VIVUS, Mountain View, CA) and has the advantage of not using a needle for delivery.

#### Testosterone replacement

Studies have shown that hypogonadal ED men show improvement in erectile function with testosterone replacement therapy. In addition, men may have improved responses to PDE-5 inhibitors in combination with testosterone replacement therapy. These men should be cautioned regarding the risks of hormonal replacement, including erythrocytosis and possible effects on the prostate. Men should be monitored while on therapy with yearly digital rectal exams, serum PSA, and complete blood counts. Any elevation in PSA or abnormal DRE should warrant prostate biopsy to rule out underlying prostate cancer. Men with a history of prostate cancer need to be cautioned about the risks of testosterone replacement therapy. Recent studies have shown that testosterone replacement therapy is safe in men with a history of prostate cancer; however, the patient should be well-informed and judicious about follow-up. Testosterone replacement therapy can be provided through injections, transdermal gels, or implantable pellets.

#### Surgical therapy for ED

Surgical therapy for ED is reserved for patients who fail medical therapy or exhibit an underlying condition that is not amenable to medical therapy. Surgical therapy involves implantation of a penile prosthesis. The prosthesis may consist of inflatable cylinders, or malleable rods. The inflatable devices consist of intracavernosal cylinders with a reservoir and a scrotal pump. In two-piece devices, the scrotal pump and reservoir is self-contained, whereas the three-piece device contains a separate reservoir that is implanted within the pelvis. These devices carry very high patient and partner satisfaction rates. Risks include infection or malfunction of the device that may necessitate removal. In certain men with documented arteriogenic ED resulting from pelvic trauma, penile revascularization surgery may be indicated. The ideal

patients are young men with no risk factors for ED and have documented arteriogenic insufficiency diagnosed with pelvic angiography. Successful revascularization in these patients can result in normal erectile function in the majority of men.

## **Summary**

Currently, most men with ED can be safely started on a trial of PDE-5 inhibitors following history, physical exam, and basic laboratory studies. Patients with a contraindication for PDE-5 inhibitor use, or who have failed PDE-5 inhibitor use, may consider VED, intracavernosal/transurethral therapy, or surgical options.

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## Is there an andropause, better known as Testosterone Deficiency Syndrome, and if so, what tissues are affected and how?

*Fertility, androgen production and sensitivity, osteoporosis and sexual function in aging men*

A. Morales

The isolation and synthesis of the testosterone molecule more than 70 years ago led to major discoveries in urology related to the fields of gender differentiation, fertility, sexual function and, of course, prostate gland development and health. Despite the many years of study, a great deal of controversy still exists regarding the diagnosis, treatment and monitoring of Testosterone Deficiency Syndrome (TDS). Figure 1 summarizes these issues and the Endocrine Society has provided a reliable guideline on this subject.

nation as well as a basic battery of tests to confirm that an insufficient level of testosterone in plasma exists. Common manifestations of testosterone deficiency include tiredness, fatigue, irritability, sexual difficulties, sleep disturbances and hot flashes. None of the symptoms can be diagnostic on its own, as other conditions such as depression and thyroid dysfunction can present a similar clinical picture. Standardized questionnaires exist to facilitate the initial assessment. However, they lack specificity to be used as a diagnostic tool. Physical signs of TDS include truncal obesity, sarcopenia and osteoporosis. Hypogonadism has been increasingly linked to the manifestations of the metabolic syndrome (MetS), a combination of medical disorders that increase the risk of developing cardiovascular disease and diabetes.

A biochemical assessment is mandatory in the diagnostic process for TDS. At the very minimum, it requires a total plasma testosterone determination. Borderline values call for a repeat determination. A low testosterone level in the presence of elevated gonadotropins is diagnostic. Although hyperprolactinemia is a rare condition, it is known to cause secondary hypogonadism. Thus measurement of serum prolactin is advisable if testosterone level is low. Occasionally, a clear clinical picture of TDS is not supported by the laboratory evaluation. Since the measurement of testosterone in peripheral blood is an inaccurate reflection of tissue levels of the hormone, it has been recommended, in this situation, to offer a 3-month trial of testosterone replacement therapy. Interpretation of laboratory results is important. Total testosterone measurement is adequate in the vast majority of cases and it is a reliable and consistent technique that is widely available. The measurement of free testosterone is not recommended because only a handful of laboratories perform this test properly. By measuring sex-hormone binding globulin and total testosterone, a simple calculation can provide the so-called calculated free testosterone or calculated bioavailable testosterone, both of which are considered a reliable reflection of androgenicity.

### Treatment

Upon confirmation of TDS and in the absence of contraindications, very few reasons are there to withhold testosterone therapy (TT). There are, currently, many choices available each one with its own advantages and drawbacks.

1. *Oral:* Testosterone undecanoate is safe but is required to be taken with a substantial amount of fat to permit adequate absorption by the gut. When this condition is met, adequate levels of serum testosterone can be reached.

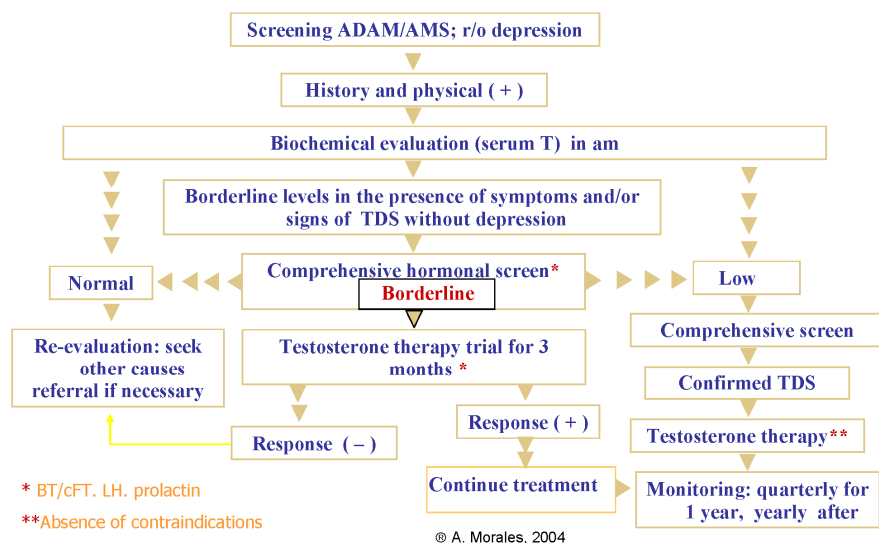


FIG. 1. Diagnostic algorithm for Testosterone Deficiency Syndrome (TDS)

### Diagnosis

The clinical diagnosis involves an adequate history and physical exami-

2. **Transdermals:** Non-scrotal patches are currently rarely used in North America because of the high incidence of skin reactions of variable severity. New patches are currently available in Europe. The manufacturers appear to have been able to circumvent the skin problems without interfering with the medication transfer.
3. **Injectables:** They constitute the earliest form of testosterone administration. Depot preparations requiring injection every 2-3 weeks are still very popular because of the low cost and the certainty of absorption. A new injectable formulation of testosterone undecanoate is available in several continents (excluding North America, at the time of writing). It requires administration every 12 weeks which is a definitive advantage.
4. **Transdermal gels:** These preparations have become increasingly popular due to the ease of administration, availability and efficacy.
5. **Non testosterone alternatives:** Under special circumstances (i.e. secondary / hypogonadotropic) TDS, a man may be a candidate for gonadotropins administration. There are also herbal, so called “phyto-androgens” of unproven efficacy. The use of dehydroepiandrosterone (DHEA) has been popular for decades but its efficacy remains highly controversial.

A few facts are worth remembering while diagnosing, treating and monitoring men with TDS:

1. The diagnosis requires a combination of clinical features and clinical low serum levels of testosterone. Not uncommonly, patients fall into a gray zone of clear symptoms but questionable biochemistry.
2. Determination of total serum testosterone is usually sufficient. Assays for free or bioavailable testosterone are properly done by just a handful of laboratories; therefore these assays are not recommended for routine clinical practice.
3. In the presence of symptoms of TDS (and absence of co- morbidities such as depression, hypothyroidism) and biochemistry results not confirming the clinical impression, we first recommended a 3-month therapeutic trial.
4. There is no evidence whatsoever that testosterone administration promotes the development of prostate cancer. But, the growth of a sub-clinical cancer might be enhanced by testosterone therapy.
5. Competent monitoring should take place every 3 months (some argue that every 6 months is sufficient) for the first year. If the patient is responding and there are no significant adverse effects, monitoring is continued on a yearly basis for the duration of treatment, which is, normally, for life.

6. Competent monitoring consist of, at least:
  - Assessment of clinical response and testosterone dose-adjustment if needed.
  - Evaluation of any possible adverse effects.
  - Physical assessment, including digital rectal examination (DRE).
  - Biochemical assessment which includes: total testosterone levels, hemoglobin and hematocrit; total, LDH and LDL cholesterol; PSA;
  - Determination of gonadotropins, other hormones (prolactin) and liver function tests are needed under special circumstances only.

## Summary

TDS occurs for a variety of reasons but aging is, probably, the most common reason for these men to seek advice. The understanding of the syndrome, its diagnosis, treatment and monitoring are not particularly complicated but demand interest and serious commitment.

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## Should there be hormone replacement therapy for aging men?

J.L. Tenover

Normal male aging is associated with a decline in serum levels of a number of hormones which have anabolic and other beneficial properties when given to young adults with established hormone deficiency. The age-related decline in these hormones parallels changes in target organs and physiological functions leading to the concept that replacing these hormones might prevent, stabilize, or even reverse some of the detrimental target-organ changes seen with aging. These hormones often have been referred to as *trophic hormones*, and include testosterone, dehydroepiandrosterone (DHEA), and growth hormone (GH). The range of potential beneficial effects of trophic hormone replacement is quite extensive and includes effects on body composition, strength and physical function, sexual function, immune function, bone density, mood, and cognition. However, to date, data from actual testosterone, DHEA, and GH replacement studies in older men have demonstrated only modest effects at best, and there are potential and observed adverse effects of replacement therapies.

### Testosterone

Total and free testosterone levels decline with normal aging in men (Fig. 1). Possible beneficial effects of testosterone replacement therapy (TRT) in older men include improvement in bone mass, body composition, strength, cognition, mood, and sexual function.

Bone mass declines with age in men, and hypogonadism is a risk factor for male osteoporosis. In general, TRT studies have shown a decline in bone degradation and increases in bone mineral density with replacement. There are as yet no data on the effect of TRT on fracture rates in older men.

Loss of muscle mass with age leads to decreased muscle strength and a decline in physical function. TRT studies in older men have consistently reported decline in body fat and increase in lean body mass (predominantly muscle mass) with therapy. The magnitude of the changes in both muscle and fat mass in older men appear similar in magnitude to that seen with TRT in young hypogonadal men. The strength changes with TRT in older men have not been as consistent as have been the changes in muscle mass, with many studies showing no beneficial effect. A meta-analysis of muscle strength changes with androgen therapy in older men reported a moderate increase in muscle strength, but one study influenced the mean effect size. In

terms of changes in physical function with TRT, only two studies to date have shown an improvement, and the magnitude of the changes in physical performance with TRT were far less than that which would be seen in response to exercise. The clinical significance, if any, of the fat mass changes with TRT therapy remain to be delineated.

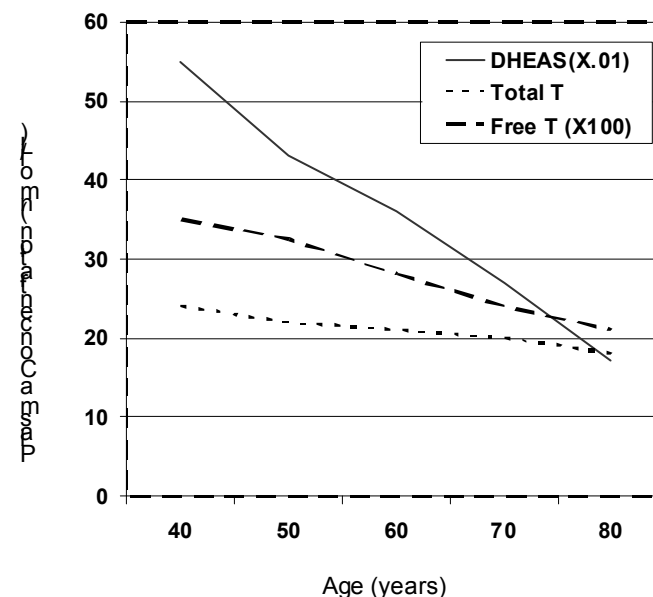


FIG. 1. Mean plasma levels of total testosterone (T), free T, and DHEAS by age in healthy men

Young men with profound testosterone deficiency have symptoms of dysphoria, fatigue, and irritability, all of which improve with normalization of testosterone levels. There have been no studies to date of TRT in depressed elderly men. In older men who were not depressed, only one of the seven clinical trials reported any mood improvement with TRT. Another five studies of testosterone therapy in older men evaluated aspects of quality of life by various scales, and none of these reported a change.

To date, there have been eight randomized controlled clinical trials evaluating cognitive function with TRT in older men without significant cognitive deficits at baseline. Half of the studies reported some improvement on aspects of cognition such as visuospatial memory, total memory, or verbal memory, and half of the studies reported no effect. There have been three small placebo controlled studies of TRT in older men with dementia. Two of

these studies reported that the TRT group did better on end of study cognitive tests than the placebo group; one study reported no difference.

The effects of TRT on aspects of the cardiovascular system are varied. Epidemiologic studies have demonstrated that low, rather than high, serum testosterone levels are associated with an increased risk of cardiovascular disease in older men. TRT studies have shown that testosterone tends to improve many cardiovascular risk parameters, such as decreasing platelet aggregation, dilatation of vessels, and improvement in total and LDL cholesterol levels. The ultimate impact of TRT on cardiovascular disease events in older men, however, is unknown.

A meta-analysis of studies of erectile dysfunction and testosterone treatment demonstrated that the prevalence of testosterone as a reversible cause of erectile dysfunction was low. TRT is not the primary treatment for the majority of older men with erectile dysfunction, but may be beneficial in some older men where decreased libido is a significant complaint, where serum testosterone levels are very low, or if testosterone therapy is used as adjunctive therapy in men who have failed PDE-5 inhibitor therapy alone (Chapter 35).

Potential or reported adverse effects of TRT in older men include fluid retention, gynecomastia, polycythemia, exacerbation of sleep apnea, and exacerbation of prostate disease. Most are rare occurrences or controllable with monitoring and with testosterone dosage adjustment. It is the potential for exacerbation of benign prostatic hyperplasia (BPH) and prostate cancer that are the main concerns with TRT. A meta-analysis of adverse events from double-blind, placebo controlled testosterone replacement trials in middle-aged and older men demonstrated that rates of prostate cancer, the number of prostate biopsies performed, and a rise in PSA were not statistically significant different between TRT and placebo treatment groups.

*Summary:* TRT improves bone density and may prevent or treat osteoporosis; however, other therapies are available. Clinically important effects of TRT on fat and muscle, such as improvement in metabolic syndrome and/or physical function, have not yet been demonstrated. TRT effects on mood and cognition are inconsistent, although it may be helpful in some older men. Cardiovascular effects of TRT may be beneficial, but there are no data on meaningful clinical outcomes such as stroke and myocardial infarction. Most adverse effects are predictable and/or manageable. The possibility of prostate cancer or BPH exacerbation by TRT remains a concern, although there are no data to support this has occurred.

## **DHEA**

DHEA(S) levels markedly decline with normal aging (Fig.1), and epide-

miological studies have reported associations between better overall health and higher DHEA levels. While early small human studies suggested DHEA supplementation might be beneficial for a variety of clinical parameters, larger randomized controlled trials of DHEA in older adults have not demonstrated any clear evidence of meaningful benefits. A recent 2-year study of DHEA supplementation in men and women over 60 years of age found no notable effects on body composition, physical performance, insulin sensitivity, or quality of life. Other randomized trials of DHEA replacement in older adults also have failed to demonstrate improvements in mood, well-being, and cognition. Several DHEA replacement studies have reported small increases in bone density, but the effects are minimal compared to established osteoporosis treatments. There have been inconsistent small effects of DHEA supplementation in older adults on immune function.

There are no reports of significant adverse effects from DHEA in the clinical trials, but these studies have utilized physiological replacement doses of high quality grade DHEA. DHEA can be metabolized to sex steroids, but deleterious effects, such as elevation in PSA or increase in prostate volumes, have not been reported.

*Summary:* DHEA supplementation may lead to small improvement in bone density, but much less than traditional therapies; there are no significant or consistent effects on any other clinical parameters. No significant adverse effects at physiological replacement doses of high quality DHEA.

## **Growth hormone**

While there are reasonable data to support GH replacement therapy in adults with documented GH deficiency and hypothalamic-pituitary disease, there are few data to support any use of GH supplementation for age-related changes in muscle, strength, bone, and other parameters. Currently, it is illegal in the United States to give GH as an anti-aging therapy.

A recent review of randomized controlled clinical studies of GH therapy reported small increases in muscle mass and decreases in fat mass compared to placebo treated older men. However, no improvements in strength, endurance, or function were found with GH therapy. Cholesterol levels tend to decrease with GH therapy, but other serum lipid levels, bone density, and quality of life did not change.

Side effects of GH therapy in older persons include peripheral edema, arthralgias, carpal tunnel syndrome, gynecomastia, and impaired fasting glucose levels. No study of GH therapy in older persons has been of sufficient duration to evaluate cancer risk.

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## What is the importance of psychosocial issues, counseling and psychotherapy in andrology?

N. Hunt and S. McHale

Many andrological problems, such as infertility, erectile dysfunction and premature ejaculation have serious psychosocial consequences for the sufferers. These consequences vary in nature and severity, but all can have subsequent effects on the management of these problems. It is thus important that they receive appropriate psychosocial support and guidance, whether that is through practical behavioural advice to the individual or the couple, suggestions for reading to enhance the understanding of the presenting problems, or the various forms of counselling or psychotherapeutic approaches to facilitate the resolution of the issues. The approach of the psychosocial support varies depending on a number of factors such as the individual(s) concerned, the nature and severity of the *physiological* problem, and the nature and severity of the *psychosocial* problem. While many andrological problems are common, e.g., it has been estimated that 20-40% of men experience premature ejaculation, not all will require treatment. Other andrological problems may disappear or resolve on their own, or through the support of partners in a relationship.

Andrological problems are very personal, and it is often very difficult for men to discuss these issues with others, even their partner. There is a range of psychosocial problems that might result from andrological problems. The most common problems relate to the loss of control, distress, anxiety and depression, that can often exacerbate the physiological condition. For instance, with depressive symptoms a person may have lowered sexual desire. Other psychosocial problems may also interact with physiological functioning, such as alcohol or drug abuse. We will focus here on the psychosocial components of various andrological disorders.

### Psychotherapy and counseling for erectile dysfunction and premature ejaculation

This is a very difficult area for men. There are similarities in the psychosocial aspects of erectile dysfunction and premature ejaculation in that they are both forms of sexual dysfunction. Whatever the initial cause of the problem, once the disorder recurs, many men experience performance anxiety, and it is this anxiety that contributes to the persistence of the disorder. Depending on the extent and severity of the problem, this anxiety

either distracts the man from focusing on arousal, or on controlling ejaculation. The performance anxiety itself thus exacerbates the physical problem. Psychotherapy is most effective when there is a psychogenic cause, but it can be beneficial even when there is an underlying physiological problem.

Couple therapy, when the man and his partner undergo therapy together is generally advisable. This can be difficult if the man is not in a relationship (hence limiting opportunities to practice the techniques discussed in the therapy session), and it is also difficult when there are relationship problems. It is important to ascertain whether the problems between the couple are caused by the dysfunction, whether they caused the dysfunction, or whether the two are unrelated.

There are a number of psychosocial obstacles when treating premature ejaculation. These include psychosocial factors such as the degree of performance anxiety or depression, partner issues, problems with the relationship, contextual variables such as the lack of privacy, and the partner's expectations of the treatment.

Cognitive Behavior Therapy (CBT) is the treatment of choice for many psychosocial problems, and it is beneficial for premature ejaculation and erectile dysfunction. It is important that the patient is able to change both his behaviors (achieving and maintaining an erection; delaying the onset of ejaculation) and his cognitions. While the initial assessment will determine the specific problems faced by the man and his partner, there is a range of common specific behaviors and cognitions that are often present. For both premature ejaculation and erectile dysfunction, the man may need to change his sexual behaviors; this involves increasing communication and resolving any interpersonal issues with his partner. There are also a number of key cognitive distortions that the man (and his partner) may have to deal with. These are linked to the behaviors described, and include: overgeneralization ("if I couldn't get aroused last night, I won't tonight"), mind reading ("I know that she thinks I am a failure"), emotional reasoning ("If I feel that it is true then it is"), all or nothing thinking (I am useless because I orgasm too quickly), and catastrophisation ("If I fail next time then my wife will leave me"). Of course the patterns of change required on behaviors and cognitions vary enormously, but these are some of the common problems. There is research relating to the role of psychodynamically inspired psychotherapy, but the evidence regarding its effectiveness is weaker than CBT.

Counseling and mutual communication with partners and healthcare providers can be useful. When the use of counseling in conjunction with sildenafil for erectile dysfunction was examined, counseling was shown to play an important role in effective treatment. Communication also has a positive impact on the management of this disorder.



## **Infertility**

It is estimated that around 10% of the population experience problems with infertility. Medical counseling for infertile couples is regularly provided both before and during treatment. But much of this is usually concerned with providing practical advice about the physiological components of infertility, such as problems with sperm or ovum, or issues relating to the method of treatment and the likelihood of effectiveness. Infertility and its treatment could lead to the psychosocial issues such as distress, loss of control and stigmatisation being ignored. CBT, as described above, can be used to deal with the psychological problems relating to infertility, and is commonly provided jointly for both partners. Most research focuses on women, but there is evidence that men are similarly affected psychologically by infertility, particularly with regard to self-esteem and inadequacy regarding their societal role. These issues should be the focus of any psychotherapy for infertile couples.

## **Conclusion**

Men with andrological disorders will commonly have concomitant psychosocial problems. Effective medical treatment may on its own, depending on the initial cause of the problem, resolve the problem. However, in many cases men may benefit from some form of psychotherapy or counseling. There are a number of different types of psychotherapy but CBT is often the most effective. Indeed, CBT is particularly effective where clear behavioral change is required, such as with erectile dysfunction and premature ejaculation. The man is able to focus more clearly on these tasks than on many more ephemeral disorders. While the focus has been on men, when dealing with psychosocial problems, it is important that the man's partner is also involved. Sometimes, the problems arise as a result of relationship issues, and if so they must be dealt with appropriately. In many cases, it is a matter of the partner being able to provide reassurance and support. Where infertility is concerned, it is particularly important for the couple to receive joint psychotherapeutic help where required. Thus, for most men, the most effective treatment for andrological disorders – depending on the cause of the problem – may be a combination of medical and psychological treatment.

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## What is BPH?

K. McVary

The prostate is located between the bladder outlet and pelvic floor. It surrounds the urethra and has functions in urinary continence and reproduction. Secretions of the prostate help to liquefy the ejaculate. Anatomically, the prostate can be divided into 'zones' (Chapter 11). BPH occurs predominantly in the transitional zone. The two primary histologic constituents of the prostate are glands and the fibromuscular stroma (the smooth muscle and connective tissue that occupy the space between glands). There is constant turnover of these components – just as there is a constant turnover of skin and many other body tissues. An imbalance in the cell renewal versus cell death with accumulation of glandular and stromal tissues results in benign prostatic hyperplasia (BPH). In contrast to prostate cancer, these glandular and fibromuscular components maintain normal differentiation and respect the natural boundaries of the prostate. These changes can be clinically detected as benign prostatic enlargement (BPE). The exact cause of BPH is unknown but is related to aging, genetic susceptibility, androgens, e.g., testosterone and dihydrotestosterone, estrogens and neurotransmitters.

The histologic findings in BPH are age-dependent and found in approximately 50% of men at age 50 and 90% at age 90. The clinical consequences of BPH are estimated to affect more than 16 million men in the US, making it the most common of all urological problems. Approximately 500,000 men per year in the United States undergo surgical therapy for BPH-related symptoms at a cost of over \$2 billion.

### How does BPH cause symptoms and how is the diagnosis made?

In reviewing the clinical aspects of BPH, it is important to define the role of BPH in the pathophysiology of lower urinary tract symptoms (LUTS). BPH is a histologic rather than a clinical diagnosis. Consequently, while many men have histologic evidence of BPH, the number who have BPH-related LUTS (i.e., clinical BPH) is much smaller. Patients present because of LUTS, not BPE per se, and it is these symptoms that should be the primary focus in diagnosis and treatment.

The urethra passes through the center of the prostate and BPH can produce bladder outlet obstruction (BOO) by reducing the size of the lumen. Mechanistically, an increase in the 'bulk' of the prostate (glandular proliferation) or 'tightening' of the prostatic muscle (fibromuscular proliferation) can

occur. It is important to recognize that, (1) not all men with histologic BPH or BPE have clinical BPH, (2) clinical BPH can occur in the absence of marked BPE and (3) the degree of BPE does not predict the severity of LUTS and vice versa. Not all the symptoms of BPH are directly related to obstruction. Symptoms may be related to changes in the bladder occurring in response to obstruction (muscular hypertrophy and instability) or age-related changes.

While there are no universally accepted criteria for the diagnosis of clinical BPH, a diagnosis can often be made with history, physical exam and a few simple tests. Evaluation is directed towards determining if the symptoms or complications of BPH are present (i.e. urinary retention, infection, bleeding, bladder stone or renal failure) and also to exclude non-BPH conditions capable of producing similar symptoms.

Treatment is symptom-driven and therefore history is paramount. Use of the standardized American Urological Association symptom index (AUASI) is the single most useful tool to gauge symptoms. The 7 self-administered questions produce a score out of 35, with moderate and severe scores falling above 7 and 19, respectively. The AUASI is not specific for BPH LUTS but is useful in stratifying men for treatment in men who have clinical BPH.

Signs of the complications of BPH, prostate volume and any features suspicious for prostate cancer should be sought on physical exam. A urine analysis and PSA are the only tests routinely recommended by the AUA. In situations where the diagnosis is unclear, measurement of a post-void residual, urodynamics may be useful.

### How is BPH treated?

Treatment is instituted in men with moderate or severe LUTS *and* who are bothered by them. The management of BPH has changed significantly over the past 20 years.

Surgical therapy is indicated in men who have moderate-severe clinical BPH in whom medical therapy has been unsatisfactory or men with a complication from BPH. Medical therapy is considered first line therapy in all other cases. Alpha-blockers and 5-alpha reductase inhibitors (5ARI) target the smooth muscle and glandular components of BPH, respectively. The alpha-1 receptor subtype is found in abundance within the prostate and bladder neck smooth muscle. Alpha-1 receptor blockade relaxes the muscle, but do not reduce prostate size. The commonly used alpha-blockers are terazosin, doxazosin, alfuzosin and tamsulosin. Alfuzosin and tamsulosin have the advantage of not requiring upward dose titration. Onset of action is rapid and they are proven to improve LUTS and flow rates. Side effects are uncommon and include hypotension, dizziness, weakness, and retrograde ejaculation. The 5ARI's, finasteride and dutasteride, address the glandular

component by the production of DHT (responsible for glandular growth) from testosterone. Prostate volume is reduced resulting in improved symptoms and flow rates over the course of several months. Side effects include breast enlargement and decreased ejaculatory volume. Alpha-blockers and 5ARI address the symptoms of BPH by different mechanisms. 'Combination therapy', the addition of 5ARI to an alpha blocker, has proven to be beneficial in men who have larger prostates and higher PSA's. In such patients, use of 5ARI's can decrease progression of symptoms and complications, such as urinary retention, whereas alpha-blockers do not prevent progression. For men who also have urgency, frequency and nocturia that are not a result of incomplete bladder emptying, anticholinergic medications (i.e. bladder relaxants) can safely be added to a treatment regimen.

The principle behind surgical therapy is to open the urinary passage by the removal or ablation of tissue. Surgery is usually done endoscopically through the urethra with no cutting of the skin. The transurethral resection of the prostate (TURP) is considered the gold standard for the surgical treatment of BPH. Innovation and evolution of surgical approaches is continuing at a high level and there are a wide variety of surgical procedures, each with variations. Other types of surgery utilize laser ablation or tissue destruction with heat or microwaves. Complications include bleeding, infection, incontinence, retrograde ejaculation and erectile dysfunction. The incidence of significant complications is low and the most severe complications are very infrequent. In general, the degree of improvement in AUASI, quality of life and flow rate are markedly higher with surgery than medication.

## Summary

BPH is a common benign condition affecting the prostate that can produce bothersome urinary symptoms in men. Not all BPH causes symptoms and when urinary symptoms are present, obstruction of urinary outflow is but one mechanism. Evaluation with standardized symptoms scores, ascertainment of the effects on quality of life and a physical exam to exclude other conditions is important. BPH is caused by an imbalance in glandular and fibromuscular proliferation, both of which are addressed by the medical treatments of 5-alpha reductase inhibitors and alpha-blockers, respectively. When medical treatment fails or when a complication of BPH ensues, there are several highly effective minimally invasive surgical treatments available that continue to evolve with time.

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## **Are some men more susceptible to prostate cancer than others and why? What are the treatments and their effectiveness? What are the possibilities for improvements in therapy?**

D.J. Parekh and I.M. Thompson

### **Are some men more susceptible to prostate cancer than others and why?**

Prostate cancer is the most common malignant neoplasm in U.S. men. The estimated lifetime risk of disease is around 17% with a lifetime risk of death of around 3%. Prostate cancer is multifactorial in origin with genetic and environmental influences playing an important role in the origin and evolution of prostate cancer. The most common risk factors associated with prostate cancer development are increasing age, positive family history of prostate cancer, and African American ethnicity. Several loci of genetic susceptibility have been identified to be associated with prostate cancer risk as are associations with mediators of infection and inflammation. Other factors believed to be associated with prostate cancer risk are androgen exposure, estrogen, hormonal factors such as insulin like growth factors and obesity.

### **What are the treatments and their effectiveness?**

Patients with localized prostate cancer are usually treated with a curative intent. The most common modalities of treatment are:

1. *Active Surveillance*: This is a suitable approach for patients with less aggressive cancer who are potentially spared of the treatment related morbidity with a trade off of missing the window of curability. Patients on this approach are followed by serial serum prostate-specific antigen (PSA) examinations, serial digital rectal examination (DRE), endorectal coil MRI if available and repeat prostate biopsy at 18-24 month intervals with a low threshold to switch to active treatment if there is an upgrading of cancer or due to patient related anxiety. Long-term data about the effectiveness of this treatment is unavailable.
2. *Surgery*: Radical Prostatectomy by an open incision or in a minimally invasive fashion with laparoscopic surgery or with the help of a surgical robot are the most commonly surgical management approaches for localized prostate cancer. In the only randomized clinical trial comparing one treatment versus another, randomizing subjects to either surgery or

observation, surgery resulted in decreased rates of metastatic disease and cancer related mortality. Though surgery results in superior cancer control, it may lead to morbidity in the form of urinary incontinence and erectile dysfunction.

3. *Radiation therapy*: Radiation therapy is an effective modality for localized and locally advanced prostate cancer. It is administered in the form of radioactive “seeds” that are implanted in the prostate under ultrasound guidance (Brachytherapy) or in the form of External Beam Radiation. To date, no prospective randomized trials have compared the efficacy of radiation therapy to surgery or to surveillance to determine the superiority of any one modality. Besides urinary incontinence and erectile dysfunction, radiation may cause bowel and urinary bladder-related morbidity.
4. *Ablative therapies*: Recently, ablative therapies in the form of Cryotherapy and High Intensity Focused Ultrasound (HIFU) have been performed for localized prostate cancer as well as in salvage settings after failures of radiation therapy. Long term results for these modalities are unavailable.

### **Issues with prostate cancer prevention**

The goal of primary chemoprevention is to decrease the incidence of a specific cancer, ideally reducing not just the risk of cancer and treatment-related side effects, but mortality as well. Prostate cancer is an attractive and appropriate target for primary prevention because of its incidence, prevalence, and disease-related mortality. The most significant event in chemoprevention of prostate cancer occurred with the publication of the results of the Prostate Cancer Prevention Trial (PCPT). This landmark study, opened in 1993, was the first large-scale population-based test of a chemopreventive strategy in men at risk for prostate cancer. In the PCPT, 18,882 men 55 years of age and older with normal findings on digital rectal examination (DRE) and a PSA level of 3.0 ng/mL or less were randomly assigned to treatment with finasteride (5 mg/day) or placebo for 7 years. Prostate biopsy was recommended if the annual PSA level, adjusted for the effect of finasteride, exceeded 4.0 ng/mL or if the DRE findings were abnormal. The primary endpoint was the prevalence of prostate cancer during the 7 years of the study, as diagnosed by either for-cause biopsies (abnormal DRE findings or PSA level) or end-of-study biopsy. The Prostate Cancer Prevention Trial demonstrated that finasteride reduces the period prevalence of prostate cancer by 24.8%, with a slightly higher risk of high-grade disease. Recent studies have confirmed that the cancers prevented by finasteride are clinically significant and that finasteride does not result in

increased incidence of high-grade cancer. These *post hoc* studies demonstrated that the higher rate of high-grade cancers was due to detection bias in the finasteride group due to improved sensitivity of PSA, rectal examination, and prostate biopsy. Other 5 $\alpha$ -reductase inhibitors such as dutasteride as well as the antioxidants selenium and vitamin E, and other agents are currently being studied in randomized trials.

### **What are the possibilities for improvements in therapy?**

Despite several current therapeutic options, treatment related morbidity remains a significant concern for patients with prostate cancer. Several possibilities may improve upon the current options for prostate cancer. The most significant improvement would be differentiating clinically indolent from aggressive cancers. Development of biomarkers, accurate imaging modalities as well as better biopsy strategies may result in improvement of the prediction of the biologic course of prostate cancer. Patients with insignificant cancers will likely receive active surveillance or focal therapy while those with more aggressive cancers will continue to receive surgery or radiation therapy.

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## What is the risk of testosterone therapy after prostate cancer?

A. Morgentaler

A currently controversial topic is whether testosterone therapy may be safely provided to men after treatment for prostate cancer (PCa). For many decades, a prior history of PCa was considered an absolute contraindication for testosterone therapy, but recent evidence suggests that such treatment may not be as risky as once assumed. Indeed, using serum prostate specific antigen (PSA) as a biochemical marker, several small case series have reported no biochemical recurrences in men following radical prostatectomy or brachytherapy, and a recent case report noted a decline in PSA in a man with untreated PCa who received testosterone therapy for two years. The impetus for reconsidering testosterone therapy in these men stems from the growing recognition of the health benefits of testosterone therapy in hypogonadal men, including improvements in energy, vitality, sexual desire, erectile function, body composition, and bone mineral density (Chapter 37), as well as the desire of hypogonadal PCa survivors for an improved quality of life.

The origin of the historical prohibition against testosterone therapy in men with a history of PCa arose from the demonstration that PCa regressed with androgen deprivation and progressed with testosterone administration. Current evidence indicates that PCa is exquisitely sensitive to changes in serum testosterone at the extremely low end of the concentration range, but becomes indifferent to changes in serum testosterone at higher concentrations.

### Testosterone therapy in men with a history of PCa

Three small case series have reported results of testosterone therapy after treatment for localized PCa. Results of testosterone therapy in a group of 7 men with undetectable PSA following radical prostatectomy revealed no recurrences, with follow-up as long as twelve years. In another study, no biochemical recurrences were observed in 10 men with undetectable PSA following radical prostatectomy. A third series reported no biochemical recurrences in 31 men treated with testosterone therapy for a median of 4.5 years following brachytherapy.

Of interest, a case report detailed a decline in PSA after two years of testosterone therapy in an 84-year-old hypogonadal man with untreated PCa. Pathology revealed a Gleason Score of 6 with cancer found bilaterally, in two

of six cores, but the patient declined treatment for his cancer. No clinical progression was noted despite a substantial increase in serum testosterone accompanied by improvement in clinical symptoms of hypogonadism.

### Relationship of high serum testosterone and PCa

The prohibition against testosterone therapy in men with a history of PCa stems from the longstanding belief that higher testosterone would lead to more rapid PCa growth. A considerable amount of evidence suggests this belief is incorrect. A landmark study involving pooled data from 18 longitudinal studies failed to find any relationship between PCa risk and serum androgen concentrations in 3886 men with PCa and 6438 men without PCa who served as controls. Specifically, men with high serum testosterone were not at any greater risk of developing PCa than men with low serum testosterone. Moreover, a meta-analysis of placebo-controlled testosterone therapy studies revealed no increased rate of PCa or other adverse prostate events in testosterone-treated men versus placebo-treated men. Furthermore, no association has been shown between high testosterone concentrations and worrisome PCa features or outcomes, such as Gleason grade, stage at presentation, positive surgical margins, biochemical recurrence, or survival. Curiously, each of these has been reported in association with *low* serum testosterone.

### Mechanism of action of androgens on prostate tissue

There are at least two likely mechanisms contributing to the androgen-indifference of PCa beyond near-castrate testosterone concentrations. One is that the androgen receptor (AR) has a finite binding capacity for androgen, with maximal binding (saturation) occurring at low androgen concentrations, approximately 4 nM (120 ng/dl). A second is the demonstration that intra-prostatic concentrations of testosterone and dihydrotestosterone appeared unchanged after 6 months of testosterone administration despite large increases in serum testosterone, suggesting that the intra-prostatic hormonal milieu may be relatively protected from changes in serum. The saturation model is supported by multiple studies in animal models, prostate cancer cell lines, and humans. For instance, castrated animals exposed to varying concentrations of serum testosterone demonstrate a steep androgen-dependent prostate growth curve, but then reach a plateau at which increasing testosterone concentrations result in no further prostate growth. Similar results are seen in the androgen-sensitive LnCaP cell line, with even log increases in testosterone or DHT producing no greater rate of cell division once a plateau

has been reached. In healthy men, no significant changes in PSA or prostate volume were noted with exposure to supraphysiologic testosterone concentrations for up to 40 weeks. These results indicate that there is a limit to the ability of androgens to stimulate prostate growth.

## Discussion

Although controlled studies of testosterone therapy in men with PCa are lacking, the limited available evidence suggests that such treatment may not pose undue risks. The historical prohibition against this treatment has been based on an assumption that PCa is sensitive to changes in serum testosterone throughout the range of testosterone concentrations, whereas current evidence suggests that the androgen sensitivity of PCa is limited to the extremely low range of serum testosterone concentrations, and becomes androgen-indifferent at concentrations typically seen in hypogonadal and eugonadal men.

Further studies are clearly needed to provide a definitive assessment of safety and risk with testosterone therapy in men who have undergone treatment for PCa. It is important to recognize the possibility that such studies may even demonstrate a *beneficial* impact of testosterone therapy on PCa outcomes, based on the association of worrisome PCa features with low serum testosterone, and experimental evidence that androgens may inhibit prostate proliferation and promote a more differentiated and less invasive phenotype. In the meantime, it is recommended that clinicians who wish to offer testosterone therapy to men with a prior history of PCa should discuss the fact that safety data are lacking, and document informed consent.

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