Handbook of Andrology

THIRD EDITION

Edited by
Bernard Robaire
Peter Chan

Preface

Since the second edition of the Handbook of Andrology, the number of PubMed listed articles related to andrology – a discipline focuses on the fundamental and clinical science on the reproductive/sexual health and pathologies of various structures specific to species that are genetically male - has doubled. The scope of topics presented at andrology meetings and in the scientific literature has also grown to encompass breakthroughs in stem cell research and epigenetics, novel approaches to male contraception, treatments for prostate cancer, awareness of the role of andrology for the LGBTQQIA2S+ community, and concerns about the effects of viruses such as COVID-19 on male reproduction.

These developments prompted the need for a third edition of this Handbook. The fundamental information provided in the second edition has been updated. We have retained the Forward Written by Philip Troen from the First Edition of the Handbook, as it provides a historical perspective on the field. While the number of chapter has increased from 41 to 64, the underlying approach to each chapter has remained as originally conceived. Each chapter is written by an expert, or a group of experts, that provides a readily understood, up-to-date highlight of the key elements in each subject area in a style that will allow trainees to be introduced to the field of Andrology and become excited about working in this field.

As for the Second Edition, we have chosen to provide an electronic only version for the Third Edition of the Handbook. With the support of the American Society of Andrology, this edition of the Handbook is freely available, not only to all members of the Society but also to anyone wishing to gain an understanding of the many facets of Andrology. 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 to save a forest of trees. We anticipate that it will be possible to provide the Third Edition of the Handbook in Chinese, Spanish and Portuguese, also on the website of the American Society of Andrology, in the future.

It is our hope that this Third Edition of the Handbook of Andrology lays the foundation for basic scientists, clinician scientists, veterinarians, healthcare professionals, trainees, and policy makers

who have an interest in acquiring a fundamental understanding of the varied aspects of andrology.

Finally, we would like to acknowledge the extensive and 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.

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Co-Editors
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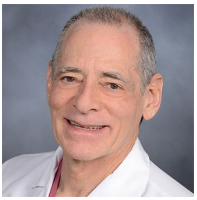

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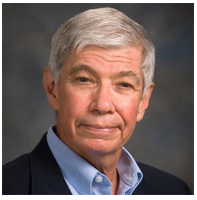

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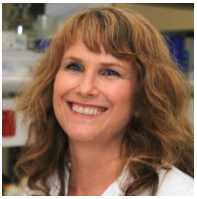

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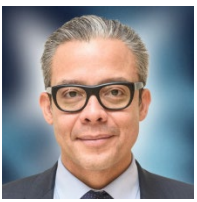

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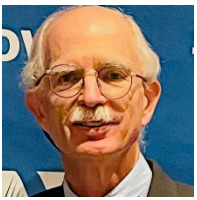

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Forward by Philip Troen

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, Comite Internacional 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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Bernard Robaire and Peter Chan

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30. What male contraceptives currently exist and what is the outlook for male hormonal contraception?

John K. Amory and Stephanie T. Page

31. What are the current approaches to non-hormonal male contraception?

John K. Amory

32. How is male infertility defined? How is it diagnosed?
Epidemiology, causes, work-up

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33. Why evaluate the infertile male in the era of ART?
Medical and surgical therapies for male infertility

Marc Goldstein

34. Can empiric medical treatments improve idiopathic male infertility?

Jared M. Bieniek and Kirk C. Lo

35. What are the assisted reproductive technologies for male infertility?
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36. Genetic factors likely cause a large percentage of male infertility

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37. What is Klinefelter Syndrome and how best to care for these patients

Samuel R. Donnenfeld and Akanksha Mehta

38. What is known about the health of ART-conceived offspring?

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39. What should I know about artificial insemination of animals? How is male fertility assessed in domestic animals and why is this important?

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40. Are sperm counts declining with time, to what extent and what are the consequences of this decline?

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42. Can spermatozoa be targets for drugs? If so, what are the consequences of such drug exposures?
Drugs that affect sperm structure or function, male-mediated developmental toxicity

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43. Can we protect germ cells against testicular insults?
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Marvin L. Meistrich

44. What types of testicular tumors occur in men? How effective are existing therapeutic approaches and what is the prognosis?

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45. What do we know about the origin of germ cell tumours?

Kate L. Loveland and Daniela Fietz

46. Can we preserve testicular germ cells of prepubertal patients for later fertility?
Next generation therapies to preserve fertility and treat infertility

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47. What is the impact of cytotoxic and surgical therapy on sperm and fertility preservation in cancer patients?

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48. What is the role of the penis in male reproduction?

Muhammed A. Moukhtar Hammad, Adnan El-Achkar, David W. Barham and Faysal A. Yafi

49. The potential reproductive effects of male circumcision

Nahid Punjani and Phillip S. Li

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

Logan B. Galansky, Phillip M. Pierorazio and Arthur L. Burnett

51. How to treat erectile dysfunction?

Wael S. Almajed, Gilad A. Hampel and Waye J.G. Hellstrom

52. Is there an andropause, more appropriately named Late Onset Hypogonadism, and if so, what tissues are affected and how?

Mitch Kim, Roei Golan, Manish Narasimman and Ranjith Ramasamy

53. Are there reproductive consequences associated with advanced paternal age?

Peter Chan and Bernard Robaire

54. Should there be hormone replacement therapy for aging men?

Austin Kinley and Mohit Khera

55. What is the place of psychosocial issues in andrology?

William Petok

56. What is benign prostatic hyperplasia and how is it treated?

Manuel Belmonte Chico Goerne, Abdulghani Khogeer, Melanie Aubé-Peterkin and Serge Carrier

57. What is prostate cancer? How is it to diagnosed? What is its prevalence?

Joseph Alukal

58. What are the treatments for organ-confined prostate cancer and how effective are they?

Partha P. Banerjee

59. How is castration-resistant prostate cancer now understood and treated?

Lara Rodriguez-Sanchez and Rafael Sanchez-Salas

60. What is an andrologist now? What does an andrologist do? What will an andrologist do in the future?

Peter N. Schlegel

61. What is the role of the andrologist with respect to the LGBTQQIA2S+ community?

Alan W. Shindel

62. How is social media changing andrology?

Heather E. Fice

63. What is the impact of COVID-19 and vaccines for COVID-19 on sexual and reproductive health?

R. John Aitken

64. Is male reproductive dysfunction a window on general male health?

Federico Belladelli and Michael L. Eisenberg

Chapter 1
What are the components of the male reproductive system?

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

Kenneth P. Roberts

The male reproductive system consists of organs acting in series to produce spermatozoa and deliver them to the female reproductive tract. The system is summarized in Fig. 1. Spermatozoa, the haploid germ cells, are produced in the testis and undergo maturational changes as they transit through 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. Finally, 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, produced in the testis, whose levels are regulated by the pituitary and hypothalamus. Knowledge of the anatomy and embryological origins of the components of the male reproductive tract is essential in developing a complete understanding of the system and its common diseases and dysfunctions.

Testis

The testis is the site of germ cell development via the process of spermatogenesis and thus the primary organ of the male reproductive system. The testis is comprised of several seminiferous tubules, where germ cell development takes place. The seminiferous tubule is comprised of Sertoli cells, the epithelial cells of the tubule, with developing germ cells populating the space between adjacent Sertoli cells. Spermatogenesis begins with diploid spermatogonia that divide by mitosis to renew the population of stem cells. A subset of spermatogonia progress to enter meiosis, at which point they are referred to as spermatocytes. After the two cell divisions of meiosis, the resulting haploid gametes are referred to as spermatids.

Spermatids morph from round cells, with the normal complement of cytoplasmic organelles, to elongated, highly specialized cells through the process of spermiogenesis. Tight junctional complexes between adjacent Sertoli cells protect the meiotic spermatocytes and post-meiotic spermatids, both of which are immunologically unique, from the adaptive immune system. Fully formed spermatids are then released into the lumen of the seminiferous tubule and transported to the rete testis, the collection site in the testicular mediastinum, for transport into the efferent ducts leaving the testis. The interstitial space in the testis, outside of the seminiferous tubules, is populated by the Leydig cells, which synthesize and secrete testosterone, and the capillary blood vessels that supply the testis.

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 scrotum forms as an out pocketing of the abdominal parietal peritoneum, called the processus vaginalis, through the anterior abdominal wall into the scrotal swellings. As a consequence, the layers of the scrotum reflect the muscle and fascial layers of the abdominal wall. The testis descends into the scrotum late in development, passing through the inguinal canal with the processus vaginalis, pulling its vasculature, nerve supply, and the vas deferens with it. The cremasteric and Dartos layers of scrotal fascia contribute to the important temperature regulatory function of the scrotum by wrinkling the scrotal skin (dartos fascia) and pulling the testes close to the abdominal wall (cremaster muscle) to conserve heat and protect the testis. Successful testis descent into the scrotum is essential for fertility, as spermatogenesis requires a temperature 2-3 degrees C lower than abdominal temperature. The processes vaginalis detaches from the parietal peritoneum to become tunica vaginalis covering the testis. Failure of the tunica vaginalis to separate from the parietal peritoneum often results in a buildup of fluid inside the scrotum which is referred to as hydrocele.

What are the components of the male reproductive system?

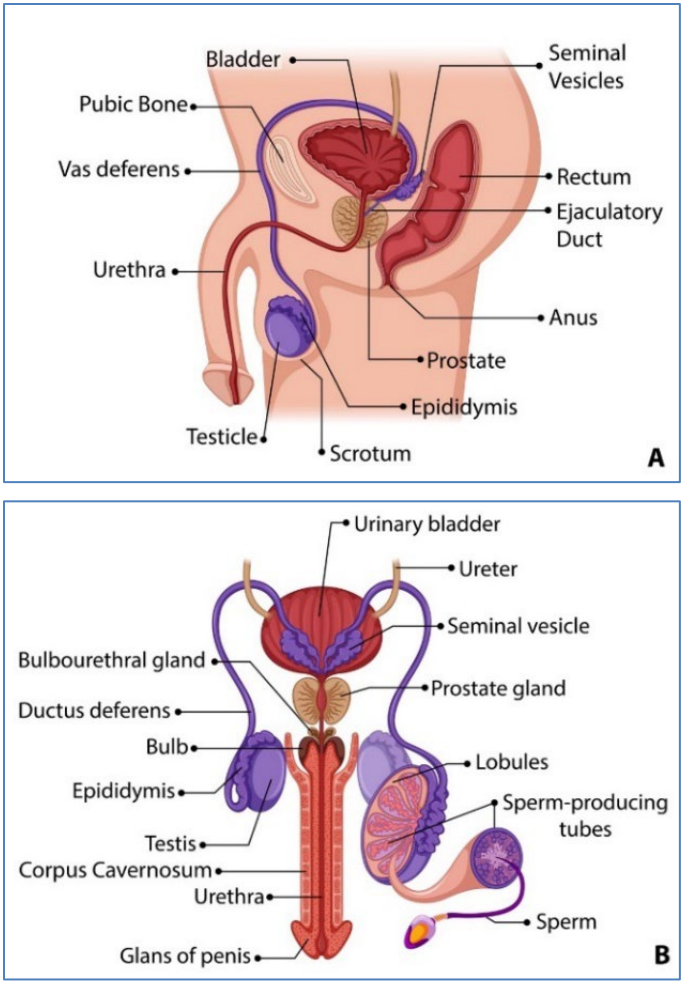

Figure 1A. Overview of the basic anatomy of the male reproductive track as it relates to the other structures of the pelvis. B. Diagrammatic depiction of the histology of the seminiferous tubules and structure of the testis, and the excurrent ducts of the male reproductive tract. (Adapted from https://www.vecteezy.com/free-vector/male-reproductive-system)

Epididymis

The epididymis is a single, highly convoluted tubule, six to seven meters long, connected to the rete testis by a series of efferent ducts.

The spermatids released from the seminiferous epithelium are immature in that they are non-motile and incapable of fertilization. The function of the epididymis is to bring testicular spermatozoa to functional maturity (Chapter 18). The maturation process includes changes in the phospholipid composition of the sperm plasma membrane and the addition and removal of specific proteins and other macromolecules to/from the maturing spermatozoa. How the epididymis accomplishes this maturation process is not fully understood. The epididymal epithelium secretes many proteins and other molecules, some packaged in exosomes, into the fluid in the epididymal lumen that bath the sperm. The components of this fluid provide the optimal micro-environment for the molecular changes that bring epididymal sperm to maturity. Mature epididymal sperm are stored in the distal part of the epididymis prior to ejaculation.

Vas deferens

The epididymal tubule ends by transitioning into the vas deferens (ductus deferens), a thickened and muscular continuation of this tubule that transports spermatozoa from the epididymis to the prostatic urethra. In its course, the vas deferens ascends from the distal epididymis in the scrotum, with the vessels that vascularize the testis and epididymis, passes through the inguinal canal, and crosses behind the bladder to enter the prostate. The ejaculatory duct, formed from the terminal portion of the vas deferens, passes through the substance of the prostate to connect the vas deferens to the prostatic urethra. The primary function of the vas deferens and ejaculatory duct is the transport of mature spermatozoa and seminal vesicle secretions (discussed below) to the prostatic urethra.

Seminal vesicles

The seminal vesicles reside immediately above the prostate gland, posterior to the bladder, and are connected to the ejaculatory ducts. The seminal vesicles are comprised of a series of tubular alveoli lined with a very active secretory epithelium (Chapter 21). 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, including semenogelin, that are involved in such processes as the coagulation of the

ejaculate and immunoprotection of the sperm when deposited in the female reproductive tract.

Prostate

The prostate gland is located in the space inferior to the bladder and superior to the perineal membrane. Its location, immediately anterior to the rectum, allows the prostate to be palpated and biopsied through the anterior wall of 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 mid-region of the prostatic urethra. The lobes are essentially continuous in the adult human prostate, with no apparent gross or morphologic distinctions (Chapter 20). A more clinically useful anatomical description of the prostate gland distinguishes prostatic zones based on morphologic and functional properties (i.e., central, peripheral, and transition zones). Certain zones are associated with specific pathologies (i.e., prostate cancer arises preferentially in the peripheral zone and benign prostatic hyperplasia in the transition zone).

Prostatic secretions contribute significantly to the fluid volume of the ejaculate (~25%). These secretions are high in zinc, citric acid, and choline and several secretory proteins, including acid phosphatase, plasminogen activator, and prostate-specific antigen (PSA). The exact roles of most prostatic secretions are unknown, although they are presumed essential for the function of spermatozoa during and after ejaculation. Many of the proteins are proteases involved in the liquification of coagulated ejaculate. An elevated level of PSA in the blood is often correlated with abnormal prostatic growth, such as cancer or benign hyperplasia of the prostate.

Penis

The penis is responsible for delivering male germ cells to the female tract during sexual intercourse. It is comprised of two corpora cavernosa and the corpus spongiosum. The corpora cavernosa are erectile tissues that when filled with blood, produce the penile erection. The corpus spongiosum, also an erectile tissue, surrounds the penile urethra and forms the glans penis. The penile urethra is continuous with the prostatic and membranous portions of the urethra and provides the remaining conduit for the sperm and

ejaculatory fluids (seminal plasma) as they leave the body. The physiology of penile erection is complex, disorders of which lead to erectile dysfunction (Chapters 50 & 51). 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), have made erectile dysfunction a primary clinical concern in andrology.

Development of the Male Reproductive Tract

The testis arises from the primitive gonad. Primitive germ cells migrate to the undifferentiated gonad from the yolk sac, causing the coelomic epithelial cells to proliferate and form the sex cords. The formation of the sex cords gives this region a raised contour called the genital ridge. During the fourth month of fetal development, 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 forming epididymis. The mesenchymal tissue in the interstitial space between the tubules gives rise to the Leydig cells, the site of androgen synthesis and secretion.

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. The distal part of the mesonephric duct forms the vas deferens and ejaculatory duct. An out pocketing of the mesonephric duct just proximal to the developing ejaculatory duct develops into the seminal vesicle. The prostate forms from a set of tubules that evaginate from the primitive urethra and not from the mesonephric duct.

Endocrine and nervous control of the male reproductive tract

The entire male reproductive tract is dependent on hormones for proper function. The pituitary produces gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), under the control of gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus. FSH acts on Sertoli cells and is required for the initiation and quantitative maintenance of spermatogenesis (Chapter 2). LH stimulates androgen production by the testicular Leydig cells. Testosterone, acting on Sertoli cells, is required in high concentration to maintain the process of spermatogenesis. The male sex accessory organs are also all dependent on androgen for proper development and 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 serially integrated nature of the system. The seminiferous tubules are continuous with the penile urethra via the epididymis and vas deferens, together comprising a tubular transit of around eight meters, 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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Chapter 2
What is the relationship among the various endocrine components of the male reproductive system?

Hypothalamic-pituitary-testicular axis-feedback loops

Ronald 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. (Fig. 1)

Hypothalamic regulation of gonadotropin-releasing hormone

Hypothalamus

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

The secretion of GnRH is regulated in the hypothalamus mainly by kisspeptin-neurokinin B-dynorphin (KNDy) neurons in the infundibular nucleus in the hypothalamus. Kisspeptin stimulates GnRH secretion directly, whereas neurokinin B stimulates kisspeptin neurons that, in turn, leads to GnRH secretion. In contrast, dynorphin has inhibitory effects on kisspeptin signaling. GnRH negative feedback from circulating levels of testosterone or its metabolic products (i.e., estradiol and dihydrotestosterone) in men inhibit the pulse frequency and secreation of GnRH. (Fig. 2)

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. The local effectors of

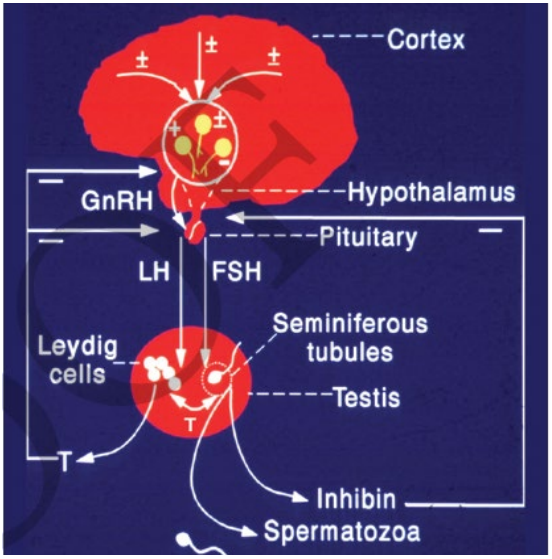

Figure 1. Schematic representation of the components of the hypothalamic-pituitary- testicular axis and of its feedback regulators.

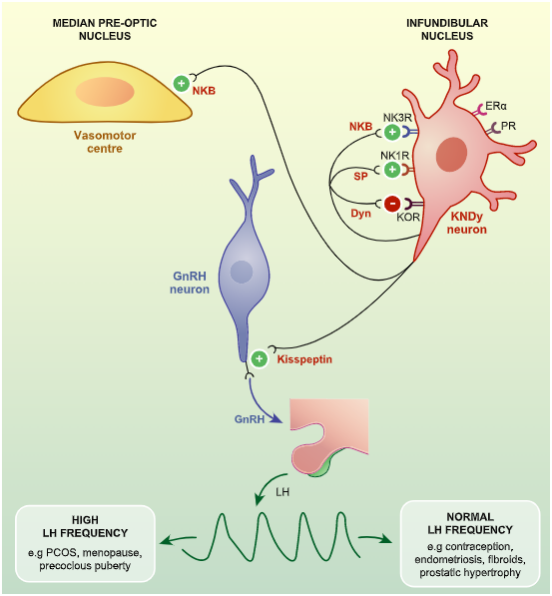

Figure 2. Hypothalamic regulation of GnRH (source: Anderson RA, Millar RP, Journal of Neuroendocrinol (2021). DOI: 10.1111/jne.13081)

GnRH synthesis and release include a number of neuropeptides, opioids, catecholamines, indolamines, nitric oxide and excitatory amino acids, γ-aminobutyric acid (GABA), dopamine, neuropeptide Y, vasoactive intestinal peptide (VIP), corticotropin-releasing hormone (CRH), and kisspeptin.

Pituitary

GnRH acts by binding to the GnRH receptors on the surface of the pituitary LH and FSH secreting cells. The normal pulsatile secretion of LH and FSH is principally driven by the pulses of GnRH from the hypothalamus. Regulation of LH and FSH levels is the result of a closed loop feedback inhibition of the hypothalamic-pituitary component by the secretory products of the Leydig cells, estradiol and dihydrotestosterone, and by inhibin secreted by the Sertoli cells. Thus, if serum testosterone is elevated, LH and FSH will be inhibited; if testosterone is low due to a primary defect in Leydig cell secretion, LH and FSH will be increased.

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. Prolactin is a potent inhibitor of GnRH secretion, thus explaining its role in inhibiting LH and testosterone secretion in the clinical condition of hyperprolactinemia.

Testes

LH and FSH circulate in the systemic blood either firmly bound to a binding protein, sex hormone binding globulin (SHBG), loosely bound by albumin or unbound (free testosterone). LH acts on surface receptors of the Leydig cells of the testes to stimulate steroidogenesis resulting in high levels of testosterone in the interstitial space and secretion of testosterone into the blood stream (Chapters 3, 4). The high levels of testosterone in the interstitial space act on the Sertoli cells in the seminiferous tubules to stimulate spermatogenesis. (Fig. 3) FSH also acts on the Sertoli cells and is required for efficient maturation of sperm that are then released into the lumen of the tubules and eventual transport with seminal fluid to the epididymis and ejaculation through the urethra.

Testosterone and its metabolites, estradiol and dihydrotestosterone, travel in the blood stream to many tissues and act on tissue receptors creating the hormonal milieu required for male sexual development and functions associated with maleness. (Fig. 4). The

testes, through their production of steroid and peptide secretory substances, provide the regulatory feedback control of the hypothalamic and pituitary components of the axis.

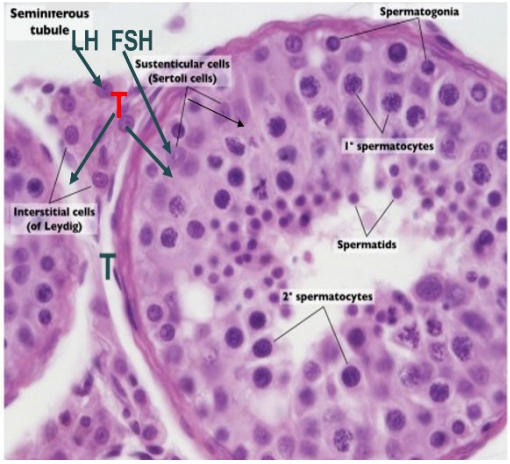

Figure 3. LH action on Leydig Cell and FSH and testosterone action on Sertoli cells.

Integration of the hypothalamic -pituitary system.

The hypothalamus through its complex neuronal network creates the pulsatile release of GnRH and the subsequent stimulation of pituitary gonadotrophs to secrete LH and FSH into the bloodstream to regulate the Leydig cells to release high levels of testosterone necessary for regulation of spermatogenesis and secretion of testosterone into the blood stream. Testosterone and its metabolites bind to cell surface and nuclear tissue receptors for end organ androgenic and estrogenic biologic actions. Regulation of the system occurs at every level, but the closed feedback effects of the steroid hormones are necessary to keep the sex steroids at physiologic levels. If there is a primary defect in hypothalamic GnRH secretion or pituitary secretion of LH and FSH then impaired steroidogenesis and spermatogenesis can occur and low levels of testosterone, LH, and FSH are measured in the blood (central hypogonadotropic hypogonadism). If the primary defect is at the testes level, then

testosterone is low and their negative feedback is reduced, GnRH, LH, and FSH increase in an effort to normalize the system. Low serum T and high levels of LH and FSH are measured in the blood (primary gonadal deficiency). (Fig. 4)

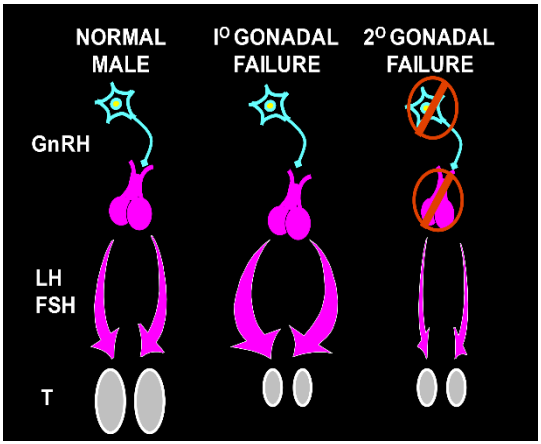

Figure 4. Primary and Secondary Hypogonadism

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Chapter 3
How are communication signals read in the male reproductive system?
Receptors for gonadotropins and androgens

Ilpo 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 the production of a variety of signaling molecules and metabolites, thereby indirectly maintaining spermatogenesis. LH exerts its action on Leydig cells by stimulating their production of testosterone (T). T, or its more potent androgenic metabolite, 5α-dihydrotestosterone (5α-DHT), stimulates spermatogenesis in concert with FSH through effects on Sertoli cell function (Chapter 2). In addition, T and 5α-DHT have numerous extragonadal actions on the differentiation, growth and mature functions of accessory sex organs (e.g. prostate and seminal vesicle). Some actions of T, e.g. on bone, occur following its conversion to estradiol (E2). Besides gonadotropins and sex steroids, an array of other hormones and growth factors, present either in the circulation (e.g. prolactin, glucocorticoids, thyroid hormone; endocrine action) or originating from neighbouring cells (e.g. various growth factors, prostaglandins; paracrine and autocrine action) exert regulatory actions on the reproductive system. 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 briefly below the cellular mechanisms of action of the two main hormonal regulators of the male reproductive system, i.e. the gonadotropins LH and FSH, and the androgen T.

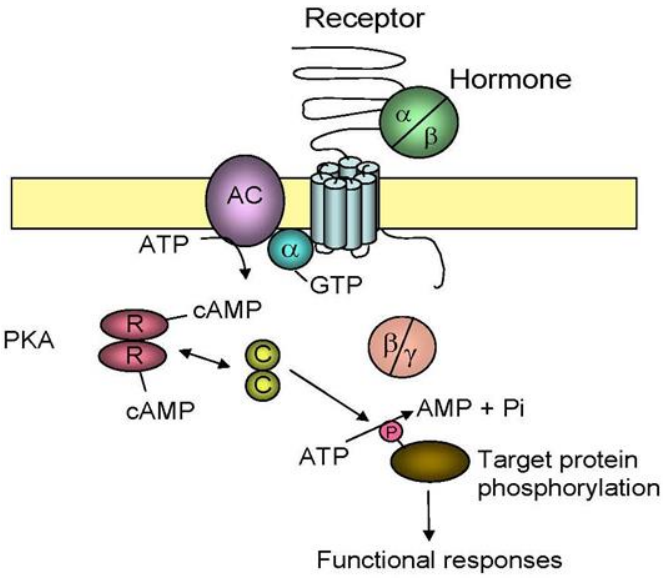

Figure 1. The mechanism of gonadotropin action. LH or FSH (Hormone) bind to the extracellular domain of their cognate receptor, i.e. LH- or FSH receptor. Both are 7-transmembrane domain G-protein associated receptors (GPCRs) and each has a long extracellular ligand-binding domain. Ligand binding induces association of guanidine trisphosphate (GTP) with the α-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 constitutes the most important functional response of target cells to gonadotropin stimulation.

Gonadotropins and Gonadotropin Receptors

FSH and LH are dimeric glycoprotein hormones secreted by the anterior pituitary gland under guidance of the hypothalamic gonadotropin-releasing hormone (GnRH). They bind to their cognate 7-times plasma membrane spanning G-protein coupled receptors (GPCR), located on the surface Sertoli and Leydig cells, respectively. The hormone-receptor contact triggers intracellularly the formation of "second messengers", of which, in the case of gonadotropin action, cyclic adenosine monophosphate (cAMP) is most important (Fig. 1). Besides the classical cAMP-mediated signalling, gonadotropins also activate other intra-cellular signalling cascades, such as calcium flux, protein kinase C, MAP kinase and PI3 kinase, but their importance in the overall gonadotropin action is still incompletely understood.

Testosterone and Androgen Receptor

Steroid hormones, including T, utilize a different principle of hormone action. Being small lipid-soluble molecules, steroids can enter their target cells through the plasma membrane. For this reason, steroid hormone receptors are located inside the cell, either in the cytoplasm or in the nucleus. Androgen receptor (AR) belongs, together with other steroid receptors, to the superfamily of ligand-activated transcription factors. Upon binding of testosterone or 5α-DHT in the cytoplasm, the ARs become dimerized, enter the cell nucleus and associate with specific DNA elements in the promoter regions of androgen target genes, thus acting as transcription factors. Another faster (within minutes) androgen action occurs through AR association with the plasma membrane and activation of intracellular kinase casades (e.g. MAPK, Akt, PKA, AMPK). The main events in the classical AR activation process are described in Fig. 2.

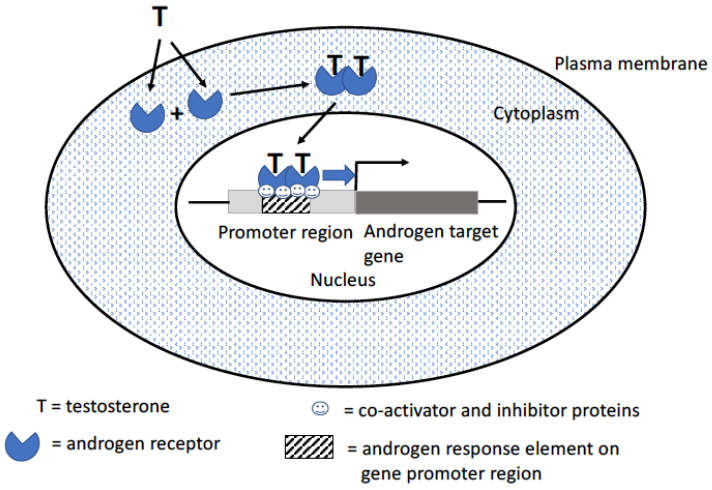

Figure 2. Mechanism of androgen action. Androgens (T) enter their target cell and bind to the cognate androgen receptor (AR), a ligand-activated transcription factor. After ligand binding AR becomes homodimerized and localized from cytosol to nucleus, where it recognizes and binds to a specific DNA motif, the androgen response element 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 involve 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.

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Chapter 4
What compounds mediate communication within the testis? Where and how are male-associated hormones produced?
Integration of the hypothalamus, pituitary and testis

Barry R. Zirkin and Michael D. Griswold

Major functions of the testis include the synthesis and secretion of testosterone, and the generation of spermatozoa. We have known for decades that testosterone is essential for the initiation and quantitative maintenance of spermatogenesis. We now know that gene expression and cell-cell interactions of spermatogenesis are regulated by testosterone and, additionally, by FSH and paracrine factors. Testosterone is produced by the testicular Leydig cells in response to luteinizing hormone (LH) from the anterior pituitary. Follicle-stimulating hormone (FSH) also is produced by the anterior pituitary. Both LH and FSH are produced in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus. Testosterone and FSH function both independently and together to regulate the proliferation, maturation and function of Sertoli cells (Chapter 2). The Sertoli cells regulate and maintain the proliferation and differentiation of developing germ cells (Chapter 7). Thus, spermatogenesis regulation involves complex interactions of the hypothalamus, pituitary and testis.

Mechanisms of action of testosterone and FSH

LH, synthesized by and released from the anterior pituitary in response to GnRH from the hypothalamus, binds to G protein-coupled receptors on the cytoplasmic membrane of Leydig cells in the interstitial compartment of the testis, thus stimulating cAMP production. cAMP stimulates the translocation of cholesterol into the mitochondria, where it is metabolized by CYP11A1 of the inner mitochondrial membrane to pregnenolone. Pregnenolone is then

converted to testosterone by enzymes of the smooth endoplasmic reticulum. It is well established that testosterone is essential for the initiation, maintenance and restoration of quantitative spermatogenesis, but that the germ cells do not respond directly to androgens. Rather, the major target cells for testosterone within the testis are the androgen receptor - containing Sertoli cells. The very high intratesticular testosterone concentration that is present in seminiferous tubular fluid greatly exceeds the concentration needed to saturate the androgen receptors. However, bioavailable testosterone is reduced by its binding to androgen binding protein (ABP) produced by Sertoli cells, a mechanism that might reduce available testosterone considerably. Just how high the concentration of free testosterone must be for spermatogenesis is unclear, and likely differs in different organisms.

FSH acts through the Sertoli cells to regulate the proliferation and maturation of germ cells. FSH binds to plasma membrane-associated G-protein coupled receptors of Sertoli cells to stimulate their proliferation and the synthesis of secreted proteins, including transferrin and inhibin. Inhibin and testosterone are involved in feedback regulation of pituitary function. FSH also plays a significant role in the initiation of spermatogenesis at puberty. Its role in 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. In the absence of FSH action, the Sertoli cell number is decreased, and this has important effects on the quantity of sperm produced. FSH and testosterone together are involved in the regulation of germ cell maturation in part by regulating adhesion complexes between germ cells and Sertoli cells, and by their impact on germ cell entry into meiosis. Thus, although FSH and testosterone have independent mechanisms of action, with FSH acting through Sertoli cell membrane-bound, G-protein coupled receptors and testosterone through Sertoli cell androgen receptors, overlapping mechanisms in their modes of action exist. Although FSH is not a mandatory requirement for the completion of spermatogenesis or fertility in rodents, its deficiency nevertheless leads to significant reduction in sperm quantity.

Paracrine regulation of spermatogenesis

The seminiferous epithelium of mammals is highly organized to assure a constant supply of spermatozoa in very large numbers. 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 the expression of Sertoli cell genes, some of which encode growth factors that 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 GFRα1. 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. 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 roles in sperm-atogenesis, if any, are uncertain. Insulin-like growth factor-1 (IGF-1) receptors are localized to germ cells, suggesting that IGF-1 may act on these cells. Transforming growth factor-α (TGF-α), TGF-β, and interleukin-1 (IL-1) are expressed, as are bone morphogenic protein 8a (BMP8a) and BMP8b. 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.

The organization of the seminiferous epithelium is initiated and maintained in part by the actions of retinoic acid (RA). RA is initially made by Sertoli cells and acts on undifferentiated spermatogonia to stimulate them to irreversibly enter into differentiation and ultimately to form spermatozoa in a series of timed molecular events. The time required to form spermatozoa is species-specific, ranging from 30 to over 70 days. To assure a constant supply of

sperm, the action of RA to stimulate undifferentiated spermatogonia is staggered and progressive along the seminiferous tubules in the form of RA pulses. As the differentiating spermatogonia progress to sperm, they move towards the lumen and overlap the germ cells developing from previous pulses. Well-defined groups of germ cells, termed stages (also known as cell associations), appear along the tubules. The stages are morphologically distinct and can be identified in histological testis tubule cross sections. The action of RA on undifferentiated spermatogonia results in the induction of key genes such as Stra8 and Kit that are necessary for the completion of meiosis, and the decreased transcription of genes such as Pou5f1, Nanos2, Lin28a, and Zbtb16 that characterize the undifferentiated spermatogonia. The enzymes involved in the synthesis of, and signaling by, RA are found in mice and in the human testis as well, but there is little information available regarding the effect of RA on human male germ cells. Clearly there are large gaps in our knowledge with respect to RA regulation of human spermatogenesis. Nonetheless, the retinoic acid synthetic pathway has been proposed both as a possible origin of some infertility and as a potential male contraceptive target.

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Chapter 5
How is the synthesis of testosterone regulated?

Samuel Garza, Martine Culty and Vassilios Papadopoulos

Testosterone, the major male sexual hormone, is secreted primarily by Leydig cells of the testis. 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, Leydig cells are considered the main source of testosterone essential for sexual differentiation and the prenatal masculinization of the male urogenital system. However, additional sources have emerged from studies in mouse, where fetal Sertoli cells were shown to generate testosterone from Leydig cell-produced androstenedione. Moreover, the existence of a "human backdoor pathway of androgen synthesis" was recently described, in which androsterone produced from placental progesterone may be as critical for human fetal masculine-ization as testosterone produced by fetal Leydig cells and dihydrotestosterone. After birth, the fetal Leydig cell 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 for 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 extents. Thus, testosterone production changes during development are due to alterations in the cellular environment and are designed for the optimal production of testosterone for specific biological needs.

How is the synthesis of testosterone regulated?

The conversion of cholesterol to testosterone is a tightly regulated process influenced by the pituitary gonadotrophin luteinizing hormone (LH). For Leydig cells to respond to LH and to function optimally, it is critical that the integrity of proteins involved in steroidogenesis, from the LH receptor to cholesterol transporting

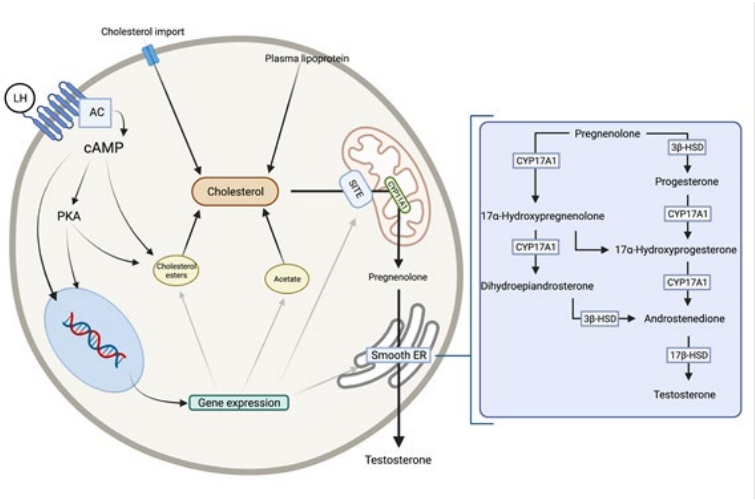

Figure 1. Schematic representation of the steps involved in testosterone formation. Luteinizing hormone (LH) binds to a G-protein coupled receptor leading to activation of adenylate cyclase (AC) that produces cAMP, the major second messenger of LH action in Leydig cells. cAMP subsequently activates the 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 transported and imported into mitochondria via SITE formed to amplify the effect of LH and cAMP. This complex included proteins such as the steroidogenesis acute regulatory protein (STAR), translocator protein (18-kDa; TSPO), and voltage-dependent anion channel (VDAC) 1. 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 3β-hydroxysteroid dehydrogenase (3β-HSD), CYP17A1 and 17β-hydroxysteroid dehydrogenase (17β-HSD) to form testosterone.

proteins and steroidogenic metabolizing enzymes, are maintained. There are several key points that are critical for the establishment and optimal function of the steroidogenic machinery responsible for testosterone synthesis in the Leydig cell (Fig. 1).

(i) Integrity of the plasma membrane LH receptor signal transduction cascade responsible for sensing and responding to the blood-borne LH. The LH receptor is a G protein-coupled receptor whose activation by LH upregulates the production of cyclic adenosine 3',5'-monophosphate (cAMP). This step precedes and initiates cholesterol mobilization and activation of transcription factors that upregulate steroidogenic genes.

(ii) Availability of sufficient amounts of the substrate cholesterol coming from the blood or synthesized de novo. Leydig cells can synthesize cholesterol de novo from acetate or source it from plasma lipoprotein, cholesterol esters, and the plasma membrane for testosterone biosynthesis. Leydig cells can also use receptor-mediated endocytic uptake to acquire lipoprotein-derived cholesterol (LDL, HDL). The de-esterification of stored cholesterol provides an ample pool of substrate for steroidogenesis.

(iii) Integrity of the mechanism responsible for transporting cholesterol from intracellular stores into mitochondria. An initial protein scaffold known as the transduceosome, comprising cytoplasmic and outer mitochondrial membrane (OMM) proteins, receives cholesterol via vesicular or non-vesicular pathways and responds to LH/cAMP. Hormone-induced proteins will join this scaffold to accelerate cholesterol import. OMM proteins further interact with proteins spanning the OMM and inner mitochondrial membrane (IMM), such as VDAC 1, mediating cholesterol loading onto the first enzyme of the steroidogenic cascade, the cytochrome P450 side chain cleavage (CYP11A1). The larger steroidogenic complex that encompasses cytoplasmic, OMM and IMM proteins is defined as the Steroidogenic InteracTomE (SITE). Some of the key proteins of the SITE include STAR, TSPO, VDAC, and 14-3-3 adaptor proteins,

(iv) Availability of appropriate levels and combinations of the nuclear transcription factors controlling the expression of proteins involved in cholesterol transport and in testosterone biosynthesis. The differential expression of steroidogenic enzymes is regulated by numerous transcription factors. These precisely regulate steroid output and Leydig cell function, preventing

either testosterone insufficiency or over production.

(v) Maintenance of appropriate organelle structures required for optimal testosterone formation. Steroidogenic enzymes reside in the mitochondria and smooth endoplasmic reticulum. The organelles' integrity and proper function are essential for normal steroid formation.

(vi) Appropriate spatial and temporal expression of steroidogenic enzymes. Cytochrome P450 monooxygenases and de-hydrogenases are responsible for metabolizing cholesterol to various intermediates leading to testosterone formation. The availability of the co-factors is also necessary for steroidogenic enzyme action.

The concepts of transduceosome and SITE have been instrumental in understanding the regulation of Leydig cell testosterone production, as well as that of steroidogenic adrenal cortical cells, which share the main steps of cholesterol transport and steroidogenic cascade. The identification of the components of the SITE complex, uncovering the spatial organization and interactions of the cytoplasmic and OMM elements, and the relationships between OMM and IMM proteins in response to hormone, have led to a better understanding of this dynamic and plastic network of proteins converging for optimal steroid hormone biosynthesis. This process can be altered by several factors. In aging, various components of the steroidogenic machinery fail to function at an optimal level, leading to a decline in androgen formation. In some cases, this can lead to significantly reduced testosterone, a condition known as hypogonadism. Indeed, the integrity of the transduceosome seems to be compromised in hypogonadism leading to Leydig cell dysfunction. The accumulation of fat mass, declining energy, alterations in mood, and decreased bone mineral density are common symptoms of low testosterone levels. Administering exogenous testosterone, testosterone replacement therapy, is commonly used to ameliorate many hypogonadism symptoms. However, monitoring and maintaining optimal testosterone levels is challenging and adverse effects have been observed, including polycythemia, peripheral edema, as well as cardiac and hepatic dysfunction. Stimulating Leydig cells to increase testosterone production is an active area of research with several identified drug targets and novel chemical entities under investigation. Stem cell-based therapy to re-establish androgen producing Leydig cells in the body has also been an active area of research for the treatment of hypogonadism.

How is the synthesis of testosterone regulated?

Inborn errors in steroid biosynthesis in the testis and the adrenal cortex are linked to mutations that can be lethal or lead to disease states such as pseudohermaphroditism, hypogonadism, and infertility. These mutations can impact numerous stages in the steroidogenic pathway, such as cholesterol transport, and steroid metabolism.

(i) Mutations in the LH receptor cause either overactivation or inactivation and disrupt the development of secondary sex characteristics. Activating mutations stimulate Leydig cells during fetal and prepubertal stages, causing autonomous testosterone production and early onset of puberty. Antiandrogen and aromatase inhibitors are effective at restoring normal prepubertal development. Mutations causing inactivation of the LH receptor result in resistance to LH stimulation and Leydig cell hypoplasia (LCH). LCH patients display varying symptoms from hypogonadism to pseudohermaphroditism.

(ii) Congenital adrenal hyperplasia (CAH) is a rare heritable disorder caused by mutations in enzymes of the steroidogenic pathway, most commonly 21-hydroxylase, and impacts nearly 1 in 5000-18000 children worldwide. CAH is characterized by reduced cortisol and aldosterone, and increased progesterone, 17-OH-prog, and sex steroids resulting in early virilization of the male.

(iii) Rare cases of 17α-hydroxylase (CYP17A1), 3β-hydroxysteroid dehydrogenase and 20,22-desmolase (part of CYP11A1) have been linked to altered androgen formation and ambiguous genitalia in boys.

(iv) Mutations in the steroidogenic acute regulatory protein (STAR), an essential protein in cholesterol transport, cause mineralocorticoid deficiency and a lipoid congenital adrenal hyperplasia phenotype among patients STAR mutations also cause CAH conditions that result in the buildup of lipid droplets in Leydig cells.

(v) TSPO mutations limit the translocation of cholesterol into the mitochondria and cause esterified cholesterol accumulations and disruptions to steroid formation

(vi) Mutations in steroidogenic factor 1, which drives the expression of many steroidogenic genes, may also result in testicular failure leading to disorders of sex development.

Testosterone production is also influenced by external factors such as drugs and environmental compounds. Numerous pharmaceuticals, agricultural and industrial chemicals act as endocrine disrupting compounds (EDCs) that can affect male reproductive functions and health, transcriptional regulation, or androgen receptor binding EDCs exposure can occur via ingestion, inhalation, or skin absorption. Bisphenols, perfluoroalkyls, phthalates, flame retardants, fungicides, herbicides and parabens, as well as dietary natural compounds have been reported to exert EDC properties on components of the steroidogenic cascade, such as LH signal transduction, cholesterol transport, and steroidogenic enzymes. The risk impact of EDCs exposure on steroidogenesis is not fully understood, given their ability to disrupt various signaling mechanisms within Leydig cells and other testicular cell types involved in male reproductive functions. Studies evaluating the impact of individual EDC's exposure on steroidogenesis may not accurately represent the risk of EDC mixtures and their metabolites, found at detectable levels in blood, such as phthalate and pesticide mixtures reported to induce reproductive defects in a cumulative manner despite individual doses being below the no-observed-adverse-effect levels.

Although the pathway of testosterone formation and its regulation by LH, as well as its susceptibility to drugs and the environment are now well established, there are many steps yet to be defined in the physiology and pathology of this complex process. In particular, the possible existence of adaptative, alternate or redundant mechanisms, especially during the developmental periods in response to environmental stressors and in aging, leave the field open for further investigations.

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Chapter 6
Are there other hormonal signals regulating testicular functions?
Role of adrenal steroids and estrogens

Maria Christina W. Avellar

Although the testes undeniably play a central role as the major endocrine organs of the male reproductive system, it is increasingly recognized that the adrenal glands and other organs/tissues of the male body contribute steroid products that are transported via the bloodstream. Once they reach the testis, for example, some of these compounds may be involved in either classic or alternate androgenic pathways as intermediates/precursors of sex hormones and other steroids. They can also exert further regulatory influence as end/branch points of synthesis by becoming a "sentinel molecule" in a feedback loop. Do adrenal androgens/androgen precursors or any adrenal steroid products have a relevant physiological function upon their delivery to the testis? The combination of experimental and clinical research is slowly but steadily unraveling these processes.

Adrenal gland and testis: focus on androgens and their steroid metabolites

In mammals, including humans, the testes and the entire male reproductive tract depend primarily on androgens for proper function. The classic and potent androgens - testosterone and its active metabolite DHT - are C19 carbon steroids. Testosterone, the main sex hormone in males, is predominantly produced in the testes and only in much smaller amounts by the adrenal glands. However, unlike other mammalian species, humans and primates are unique in having an adrenal cortex (zona reticularis) that produces and releases large amounts of DHEA (dehydroepiandrosterone) and its sulfated form DHEA-S (in addition to C19 carbon steroid products such as androstenedione and 11OH-androstenediol) into the bloodstream. An overview of the steroidogenesis pathways that take place in the adrenal glands for the production of androgens and other steroid hormones is outlined in Fig. 1. The major

intermediates/specific enzymes and cofactors of each androgenic pathway, as well as active metabolites of testosterone (DHT and estradiol) are also shown.

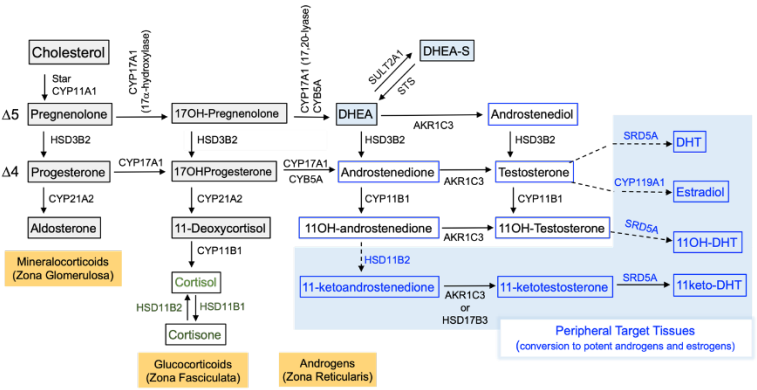

Figure 1. Overview of steroid biosynthetic pathways in human adrenal cortex. Adult cortex produces three distinct classes of steroid hormones: mineralocorticoids (ZG), glucocorticoids (ZF) and androgens (ZR). All cortical steroids are synthesized from cholesterol. The key enzymes involved in cortical steroidogenesis are either cytochrome P450 enzymes (CYPs) or hydroxysteroid dehydro-genases (HSDs). While Star and CYP11A1 are ubiquitously expressed within the adrenal cortex, the zonal segregation of other enzymes accounts for the compartmentalization of the end point steroids produced. The human adrenal ZR promotes efficient DHEA production through the Δ5 pathway; the low expression of HSB3B2 favors biosynthesis toward DHEA. CYP17A1, which competes with HSD3B2 for pregnenolone, catalyzes the conversion of pregnenolone to C19-androgens (i.e., DHEA and androstenediol). CYB5A is an allosteric enhancer of the 17,20-lyase activity of CYP17A. SULT2A1 converts DHEA in DHEA-S. STS is the primary enzyme involved in steroid desulfation. Catalytic activities of the enzymes AKR1C3 and the 11β-hydroxylase CYP11B1 produce 11-oxygenated androgens and their precursors. Conversion of adrenal steroid precursors to more potent androgens or estrogens takes place in target cells in the testes and other tissues (shown in blue background). The interconversion of active glucocorticoids (cortisol) into inactive cortisone is dependent on the tissue/cell

enzyme activity profile of HSD11B2 and HSD11B1, respectively; highlighted in green). Abbreviations: StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 cholesterol side-chain cleavage; HSD3B2, 3β-hydroxysteroid dehydrogenase, Δ4/Δ5-isomerase type 2; CYP17A1, 17α-hydroxylase/17,20-lyase; CYB5A, cytochrome b5 type A; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; AKR1C3, 17β-hydroxysteroid dehydrogenase type 5; SULT2A1, steroid/DHEA sulfotransferase type 2A1; CYP11B1, 11β-hydroxylase; T, testosterone; DHT, dihydrotestosterone; 11OH-T, 11β-hydroxytestosterone; 11OH-DHT, 11β-hydroxydihydrotestosterone; SRD5A1 and SRD5A2, 5α-reductase type 1 and type 2; CYP19A1 (cytochrome P450 aromatase); HSD11B2, 11β-hydroxysteroid dehydrogenase type 2; 11OH-androstenedione, 11β-hydroxyandrostenedione; Med, adrenal medulla; STS, steroid sulfatase.

In contrast to testicular androgens, that are produced in Leydig cells under the control of the hypothalamic-pituitary-testicular axis (Chapters 3, 5), adrenocortical-derived androgens are produced in the adrenal cortex (zona reticularis; ZR) under the control of the hypothalamic-pituitary-adrenal axis, involving CRH (corticotropin-releasing hormone) from the hypothalamus and ACTH (adrenocorticotropic hormone) from the pituitary (Fig. 2).

Whereas the enzymatic machinery yields canonical pathways Δ5 (pregnenolone→DHEA→androstenedione or androstenediol), and to a lesser extend Δ4 (pregnenolone→17OH-progesterone→androstenedione) of cholesterol-derived steroids in the testis to produce testosterone (Chapter 5), in the adrenal ZR a different but similar set of enzymes produces abundant amounts of Δ5 DHEA and DHEA-S, followed by androstenedione and 11OH-androstenedione. The limited HSD3B2 expression but increased expression/activity of the 17,21-lyase function of CYP17A1 and of its allosteric regulator (cytochrome b5) favors DHEA and 11OH-androstenedione production over androstenedione/testosterone pathway synthesis in cortical ZR. In turn, high levels of sulfotransferase SULTA1 converts most of the nascent DHEA to DHEA-S for secretion. This sulfated form has a longer half-life in the blood, resulting in higher circulating concentrations compared to the non-sulfated forms. DHEA and DHEA-S not only serve as precursors for other steroids but can also serve as important biomarkers in themselves to help differentiate among different diseases of androgen excess. In lieu of the testicular enzyme

17β-HSD3, the human adrenal ZR employs AKR1C3 for the production of either androstenediol or testosterone. In addition, the ZR also produces 11-oxygenated steroids and their precursors through the catalytic activity of AKR1C3 and other adrenal specific enzyme CYP11B1. Only a small fraction of total circulating testosterone and about half of circulating androstenedione are of adrenal origin.

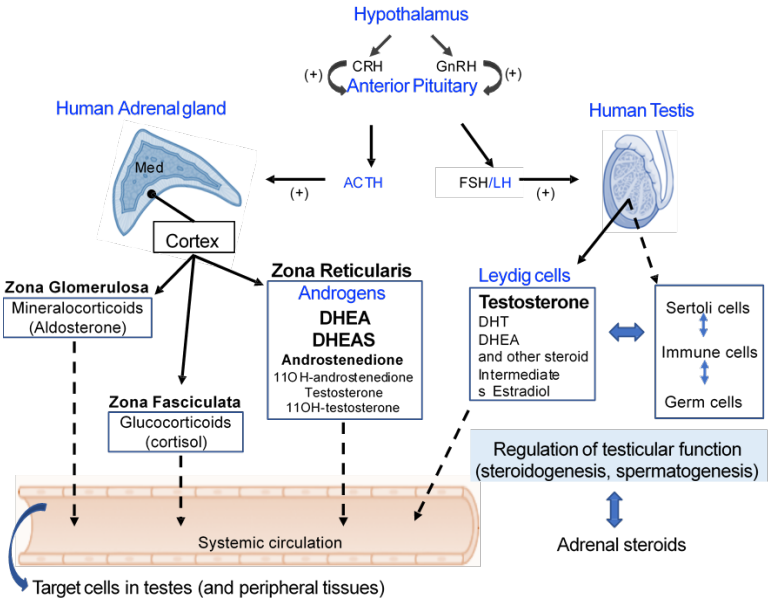

Figure 2. Schematic representation of the HPA axis components controlling steroidogenesis in the human male adult adrenal cortex and testis. Focus is given on the production of androgens and precursors in both endocrine glands. Adrenal and testicular steroidogenesis is under hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonad (HPG) axis modulation, respectively. In adult men, most of the circulating DHEA and almost all DHEA-S is of adrenal cortex origin (ZR); a portion of circulating DHEA/DHEA-S is produced by the testes. Once adrenal steroids reach the systemic circulation, they can act on target cells as they are, or they may be further converted to more potent androgens inside those cells.

Uptake of circulating DHEA-S occurs via membrane transport proteins expressed in testes and other tissues (organic anion transporting polypeptides; OATPs); its sulfate must then be removed

before it can be converted to androstenedione in target tissues. Desulfation is driven by sulfatases that are active in these tissues before further intracrine conversion into more potent androgens (testosterone/DHT) and/or estrogens (e.g., estradiol) which then exert their effect in target tissues via their cognate receptors (AR and ER, respectively). Compared to testosterone, androstenedione and DHEA have relatively little androgenic effect on their own; on the other hand, the adrenal-derived 11-oxygenated androgens, for example, are bioactive androgens, i.e., can induce androgen effects through AR activation.

In retrospect, it is easy to see that the adrenal gland is capable of making products that can be inserted into testicular steroidogenic pathways, because the testis and the adrenal gland have a common embryological origin within the urogenital ridge. They share common genetic and steroidogenic properties that contribute to steroid/androgen production in a developmentally specific manner throughout life. Adrenal androgen secretion is directly dependent on *de novo* steroid production, because there are no stored intermediate reservoirs in the adrenal cortex. The fetal adrenal gland is proportionally larger than the adult tissue, consisting mainly of the fetal zone (FZ), which resembles the ZR of the adult cortex (Fig. 3). Until birth, it secretes large amounts of DHEA and DHEA-S; one critical physiological role, after their intracrine conversion to estrogens, is to support the formation of the fetal-placental unit, thereby maintaining fetal development and pregnancy. After birth, the FZ regresses and DHEA-S synthesis decreases in the first months of life. Production of androgens by the ZR of the adrenal begins at adrenarche (~6-9 years of age in boys), just prior to puberty onset (Chapter 28). This increase in adrenal-derived androgens (primarily DHEA-S) during adrenarche serves as a precursor and possible signal for the synthesis of the more robust androgens generated through target tissue intracrine pathways. From puberty to adulthood, production of androgens by the adrenal glands becomes more closely integrated with testicular androgen steroidogenesis.

Testicular and adrenal testosterone is metabolized to DHT in the testes and other target tissues with cells expressing the enzyme 5α-reductase (SRD5A). Testosterone, but not DHT, is converted into estrogens in cells expressing the enzyme aromatase (CYP19A1). Estrogens are another group of active steroid hormones important for testicular function and male fertility. Steroidogenic precursors are also stored and secreted in smaller amounts by the testes. Once

they reach a cell with the specific enzymatic repertoire, both testicular testosterone and adrenal/testicular androgen precursors can be converted to active androgens and/or estrogens locally in target tissues (intracrine mechanisms).

Estrogens

The next steroids exerting effects in the male reproductive tract in mammals, are estrogens, chiefly estradiol. Under LH stimulus, testosterone and androstenedione are converted to estradiol and estrone, respectively, by CYP19A1 aromatase. In human testes, CYP19A1 is localized in Leydig, Sertoli and germ cells. Throughout the male lifespan, Sertoli cells are considered the earliest site of estrogen production in the testis, switching to Leydig cells during neonatal development, when gonadotropin-regulated aromatase is present. In contrast, Leydig cells are the primary site where testosterone is converted to estradiol in the adult.

In the case of estradiol, the source of estrogen can be both the cell itself, which exerts the estrogenic effect, or the traditional remote endocrine mechanism via the bloodstream. Estrogenic effects, both in reproductive and non-reproductive tissues, are exerted through at least three different estrogen receptors: ESR1 (ERα), ESR2 (ERβ), along with GPER (G protein-coupled estrogen receptor). They are abundantly expressed in testicular cells and throughout the male reproductive tract. Aromatase in human sperm allows androgens to be converted to estrogens as they pass through the reproductive tract, yielding free estrogens in seminal fluid that can act on other male reproductive organs.

A balance of intra-testicular testosterone and estrogen levels is critical for normal testicular function (steroidogenesis and spermatogenesis). In men whose spermatogenesis is impaired by Leydig cell dysfunction, there is usually increased aromatase activity resulting in elevated intra-testicular estrogen levels and a decreased testosterone/estradiol ratio. Increased aromatase activity and estrogen levels in male adipose tissue are associated with obesity and a decrease in fertility. Disturbances in aromatase or estrogen receptor activity have varying effects on fertility, ranging from minimal transitory disturbances in sperm count and function to permanent loss of fertility. In summary, testes are capable of synthesizing and responding to estrogens throughout their development.

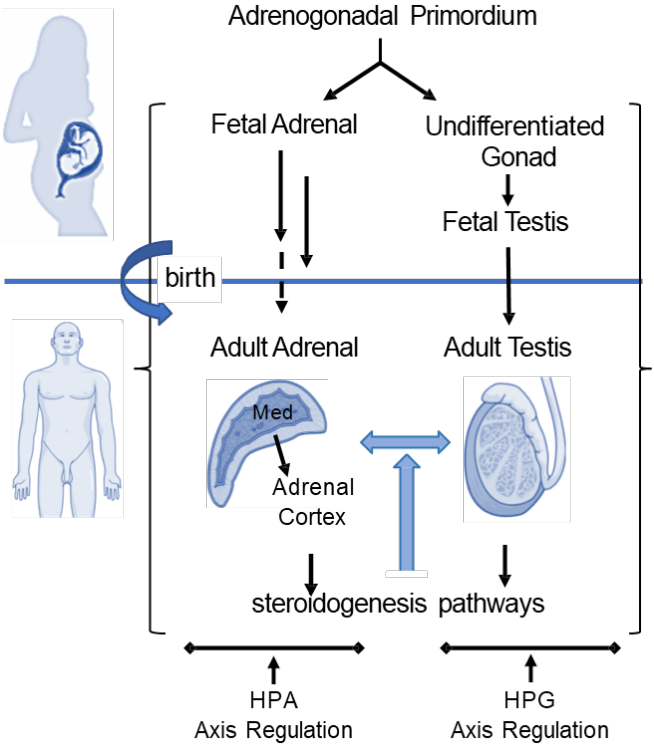

Figure 3. Human male adrenal gland cortex and testis development. The adrenal cortex and testis have a common embryonic precursor in early embryogenesis and subsequently they have common biological processes, such as the ability to produce steroid hormones. Steroid production starts in both tissues during fetal life. In the case of the adrenal gland, initially the fetal tissue consists of a large fetal zone (FZ) that secretes abundant androgen precursors DHEA/DHEA-S. Later on, neural crest-derived chromaffin cells migrate through the fetal cortex to form the future medulla. Next, the newly formed transition zone starts producing cortisol. Shortly before birth, mineralocorticoids are also secreted by the tissue. After birth, the FZ regresses, and the adrenal cortex restarts steroid production/secretion just prior to puberty. In the adult adrenal, the cortex zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) are responsible for producing mineralocorticoids (aldosterone), glucocorticoids (cortisol) and androgens (mainly DHEA/DHEA- S), respectively.

Glucocorticoids

The adrenal cortex (zona fasciculata, ZF) also produces and secretes glucocorticoids (cortisol in human) which, like estrogens, are important regulatory steroid hormones for the development and maintenance of testicular function, male reproductive health and fertility. In adults, glucocorticoid excess has been demonstrated to adversely affect LH-Leydig cell communication through glucocorticoid receptors (GR) expressed in hypothalamic neurons, pituitary gonadotrophs, as well as in Leydig cells themselves. Taken together, these glucocorticoid-induced effects regulate the concentration and efficiency of the steroidogenic enzyme machinery of the Leydig cells. The access and biological effects of cortisol on target cells in the testis is controlled by local relative expression and activity of the isoenzymes 11β-HSD2 and 11β-HSD1 which catalyze the interconversion of active cortisol to its inactive metabolite cortisone and vice-versa, respectively. GR expression is also present in other types of human testicular cells, including Sertoli cells, immune cells (e.g., macrophages) and possibly in germ cells. There is growing evidence from animal models for the ability of testicular immune cells to synthesize their own glucocorticoids, more specifically in macrophages adjacent to Leydig cells; this testicular-immune cell source of glucocorticoids opens new avenues for analogous processes in humans.

Summary

While the testis exhibits a dominance over the male steroid synthesis pathways, other somatic sites of steroid synthesis (e.g., adrenal cortex) contribute other androgens and non-androgen steroids that modulate the diverse steroid pathways of the testis. The development of more sensitive and precise assays for characterizing adrenal and testicular steroid molecules earlier thought of as only precursors or inactive intermediates, is uncovering larger and more important roles for these compounds. Previously relegated to playing minor parts in androgenic processes, these newly recognized steroid players can now be analyzed to better assess their contributions to the biosynthetic pathways, signaling cascades and regulatory processes relating to male reproduction. These emerging androgenic participants from both within and without the male reproductive tract now offer growing opportunities for examining the molecular crosstalk in this burgeoning network of testicular processes. As we move forward, it is the increased sensitivity and

precision of the technologies used to elucidate the recently demonstrated roles of these androlological molecules that can now be harnessed to develop diagnostics to aid in the differentiation of pathologies resulting from disruptions among these steroid players and their associated pathways.

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Chapter 7
What are the multiple roles of the Sertoli cell?
Supporting spermatogenesis and immune regulation

Jannette M. Dufour

Introduction
The primary function of the testis is to produce testosterone and sperm. The testis is divided into two compartments: the interstitium and the seminiferous tubules (Fig. 1). The interstitium is located between the seminiferous tubules and contains Leydig cells, immune cells, blood vessels, and lymphatic vessels. Leydig cells are responsible for producing the male sex hormone testosterone, which is secreted from the testis and is critical for the development of male external genitalia and initiation of spermatogenesis. Sperm production occurs in the seminiferous tubules. The seminiferous tubules contain Sertoli cells and germ cells and are surrounded by peritubular myoid cells (Fig. 1). Sertoli cells are sustentacular cells that were first described by Enrico Sertoli in 1865. They extend from the base to the lumen of the seminiferous epithelium and have been described as nurse cells as they engulf the germ cells and provide an appropriate microenvironment for germ cell development.

At puberty, testosterone levels rise, triggering the onset of spermatogenesis. At this time, the spermatogonia progress to primary spermatocytes and the advanced germ cells (spermatocytes and spermatids) first appear (Fig. 1). During spermatogenesis, germ cells undergo three stages of development: mitosis (spermatogonial proliferation), meiosis (spermatocyte DNA recombination, reduction and division) and spermiogenesis (spermatid differentiation). After differentiation the sperm are released into the lumen and residual bodies (containing excess spermatid cytoplasm) are phagocytosed by the Sertoli cells. Since the advanced germ cells are not present at birth when immune self-antigen tolerance is established, there is potential for an immune response to be generated against the germ cells that could result in autoimmune orchitis (testis inflammation and anti-sperm antibodies), loss of germ cells and male infertility.

Role in spermatogenesis

In addition to providing structural support and creating the appropriate environment for germ cell development, Sertoli cells provide a plethora of factors important for spermatogenesis including growth factors, hormones, metabolites, nutrients, adhesion molecules, proteases, retinoic acid, and iron to name a few. The precise time when these factors are produced is important for maintaining the cycle of the seminiferous epithelium.

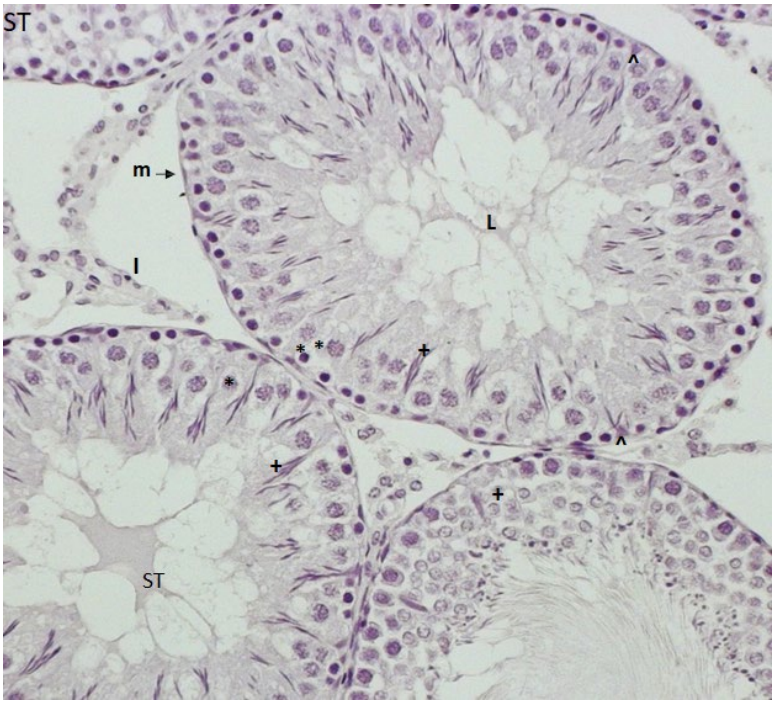

Figure 1. Organization of the testis. The testis is divided into the seminiferous tubules (ST) and the interstitial space (I). The seminiferous tubules are surrounded by peritubular myoid cells (m) and contain the germ cells and Sertoli cells (caret symbol points to Sertoli cell nuclei). *, spermatocyte; +, round and elongated spermatids; L, lumen.

The various different germ cells present in the testis are organized into the cycle of the seminiferous epithelium, which is defined by the specific germ cells present in a given cross section of the tubule. The timing of this cycle varies between species and is controlled by the

germ cells. This was shown by transplantation of rat germ cells into mouse testes, which led to incorporation of the timing of the rat cycle in mice thus demonstrating that the germ cells are responsible for the timing of the cycle of the seminiferous epithelium. Consistently, at least in mice, Sertoli cells are present in two different transcriptional states that are dependent on the presence of germ cells. These states correlate with high and low retinoic acid levels and suggest that germ cells control the timing of the expression of specific factors by Sertoli cells that are necessary at specific times of their development.

Besides their roles in testis formation during embryonic development, cell ablation studies confirmed the role of Sertoli cells in testis and germ cell development postnatally. Loss of Sertoli cells led to loss of all germ cells and spermatogenesis. Additionally, Sertoli cells are necessary to maintain the differentiated state of peritubular myoid cells before but not after puberty and for peritubular myoid cell function in the adult. Sertoli cells are also necessary for adult Leydig cell development and survival. These studies further support the critical role of Sertoli cells in spermatogenesis and expand their importance to regulation of the other testicular cells.

Physical, physiological and immunological components of the blood-testis-barrier

The blood-testis-barrier (BTB) is also formed at puberty. However, unlike other blood tissue barriers, the BTB is not located at the blood vessels but instead is located within the seminiferous tubules toward the basal side of the Sertoli cells. It consists of the body of the Sertoli cells and the tight junctions formed between adjacent Sertoli cells and separates the seminiferous epithelium into the basal and adluminal compartments (Fig. 2).

The BTB has physical, physiological and immunological functions that work together to prevent germ cell exposure to toxins, create the appropriate milieu for germ cell development and prevent an autoimmune response to the advanced germ cells. The physical and physiological components act together to control the movement of molecules across the barrier. The physical barrier restricts the passage of some molecules, while the physiological part contains specific transporters located along the basal and apical membranes of the Sertoli cells and regulates the movement of factors in or out of the lumen. In this way the Sertoli cells are able to

create the appropriate environment for germ cell development. Additionally, the immunological part functions to prevent entry of antibodies and, together with the peritubular myoid cells, the BTB prevents entry of immune cells into the seminiferous tubules.
One interesting aspect of the BTB is the need to both protect the germ cells from an immune response (and maintain the microenvironment for germ cell development), and yet at the same time allow the germ cells to cross from the basal to adluminal

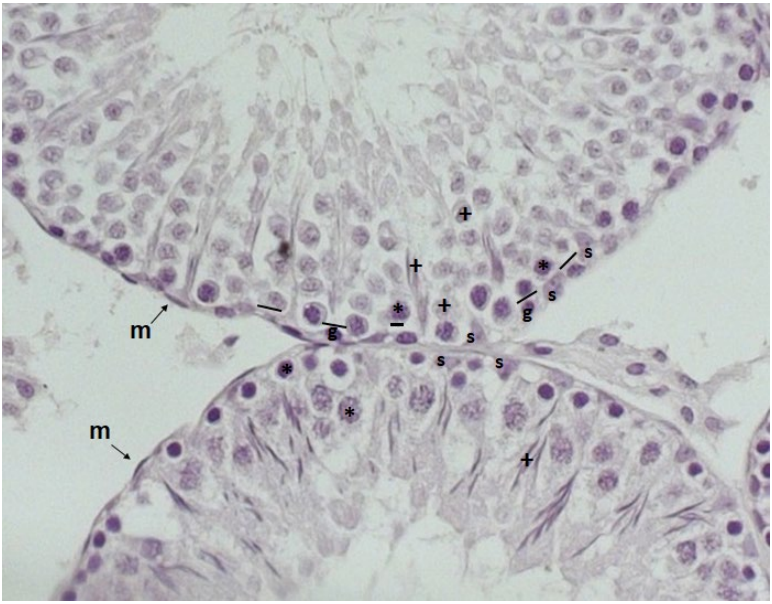

Figure 2. Seminiferous epithelium. The BTB (lines represents TJ) between adjacent Sertoli cells separates the seminiferous epithelium into the basal (containing spermatogonia and preleptotene spermatocytes) and adluminal (containing spermatocytes and spermatids) compartments. m, peritubular myoid cells; s, Sertoli cell; g, spermatogonia; *, spermatocyte; +, round and elongated spermatids.

compartment without exposing the contents of the adluminal compartment to the immune system. This process requires unique coordination. As the preleptotene spermatocytes progress to leptotene spermatocytes, they migrate away from the basal edge of the tubules. At this time, new tight junctions form below them, creating an intermediate compartment with the old tight junctions above and new tight junctions below. This process is partially

coordinated by testosterone and cytokines such as TNFα and TGFβ 2 and 3. Testosterone promotes the formation of new tight junctions below the spermatocytes, while the cytokines disrupt the BTB causing the disassembly of the old tight junctions. This transient intermediate compartment allows the spermatocytes to cross the BTB without opening the adluminal compartment to the interstitial space. Consequently, the BTB is a dynamic barrier, constantly breaking down at some points and being remade at others.

Sertoli cell as immune regulators

Given the immunogenic nature of the germ cells, the testis must be able to prevent an immune response to protect the germ cells. At the same time, bacteria and viruses can infect the testis, and therefore the testis must also be able to mount an antimicrobial response to prevent infections. However, if the inflammation associated with the antimicrobial response is not held in check, it can lead to an autoimmune response and male infertility. Conversely, if the testis fails to mount a robust antimicrobial response, the testis can become a sanctuary site for pathogens. While numerous components allow the testis to both provide germ cell protection and inhibit infections, here we will focus on the role of the Sertoli cells.

The initial evidence for Sertoli cell involvement in testis immune regulation comes from transplantation studies. Transplantation of tissue from genetically different individuals (allogeneic) or from a different species (xenogeneic) usually results in immune rejection of the grafted tissue within a few weeks. On the contrary, when allogeneic or xenogeneic tissue is transplanted into the testis, it enjoys prolonged survival. Elimination of Leydig cells and germ cells demonstrates the importance of Sertoli cells in this immune protection as foreign tissue grafts still survived long-term in the absence of Leydig cells or germ cells. Moreover, transplantation of isolated Sertoli cells as allografts or xenografts to an ectopic site outside the testis, had long-term survival without the need for immune suppressing drugs. Also, co-transplantation of Sertoli cells with allogeneic or xenogeneic tissue, such as pancreatic islets, prolongs the survival of the co-grafted cells. Lastly, ablation of Sertoli cells from the testis showed that Sertoli cells are required to form the BTB, which is necessary to prevent the entry of antibodies and immune cells into the adluminal compartment of the seminiferous epithelium, although the remaining peritubular myoid cells were capable of blocking the entry of macrophages into the tubules.

As mentioned above, one mechanism to prevent an immune response is to sequester the germ cells behind the BTB. However, there is more to immune protection of the germ cells. Recently, it was found that not all germ cell antigens are sequestered and instead some antigens are localized to residual bodies where they are phagocytosed by Sertoli cells, exported from the seminiferous tubules and present in immune complexes in the interstitial space. This leads to induction of regulatory T cell (Tregs) immune tolerance. Tregs were found to be critical for this tolerance as Treg depletion resulted in autoimmune orchitis. In addition, Sertoli cells express several immunomodulatory factors that can alter the immune cell response towards tolerogenic. For instance, they express immunoregulatory factors (TGFβ, IDO, activin A, IL6, galectin 1, IGF1), complement inhibitory proteins (C1INH, CD46, CD55, CD59 and clusterin), and anti-apoptotic proteins (serpina3n, serpinb9). These factors can influence macrophages (M2), T cells (Tregs) and dendritic cells (tolerogenic DC) to be immune protective. For example, Sertoli cell expression of galectin 1 has been implicated in the production of tolerogenic DC that in turn induce Tregs and Sertoli cell production of TGFβ and IDO has also been connected to Treg induction.

Under other circumstances, Sertoli cells can shift to an antimicrobial response and express inflammatory mediators to fight off infections. Sertoli cells express inflammatory cytokines (IL1β, IL6, TNFα, MCP1), pattern recognition receptors (TLR 2, 3, 4, 5, and 6, NOD 1 and 2), NFκB and antimicrobials (β-defensin, α-defensin, PKR). In response to viruses and bacteria, pattern recognition receptors initiate signaling cascades like NFκB. NFκB activation leads to increased expression of the inflammatory cytokines and antimicrobials mentioned above, which promotes immune cell migration and activation (M1 macrophages, dendritic cells, Th1 and Th17 CD4 T cells, and CD8 CTL T cells). Sertoli cells can also express TAMs (Tyro3, Axl and Mer). TAMs suppress TLR signaling pathways and decrease inflammation. Together this allows the Sertoli cells to provide the delicate balance necessary to protect the germ cells and fight off pathogens.

Bacteria and at least twelve viruses have been detected in the testis. Several bacteria were found to infect the male reproductive tract but it is very rare for bacteria to reach the testes. In contrast, viral infections are much more common and it has now been confirmed that the testis can act as a viral sanctuary site for viruses that can be sexually transmitted. Not only is this a concern from an

infection stand point but viral infection induces an inflammatory response that leads to germ cells loss. Marburg, Zika, mumps, HIV, and SARS-CoV-2 can infect testicular cells, and all except HIV, and SARS-CoV-2 have been shown to infect Sertoli cells. Persistent viral infection activates TLRs and initiates an inflammatory response that alters BTB TJ proteins and increases BTB permeability, ultimately leading to loss of germ cells. Although rare, the testis can also serve as a reservoir site for relapse of acute lymphoblastic leukemia. Together this emphasizes the need to increase our understanding of testicular, including Sertoli cell, immune regulation.

Conclusions

Sertoli cells play important roles in supporting spermatogenesis and controlling testicular immune regulation. They provide the structural support for the three stages of germ cell development and create the BTB with physical, physiological and immunological functions. This allows them to maintain the appropriate milieu necessary for germ cells to progress through meiosis and spermiogenesis. At the same time Sertoli cells regulate the immune response to both protect germ cells from the immune system and also provide an antimicrobial response to prevent infection. If this is not properly controlled, it can result in an autoimmune response against the germ cells or provide a sanctuary site for pathogens.

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Chapter 8
What are male germline stem cells?
Factors controlling germ cell production

Makoto Nagano

A man produces sperm at the rate of ~1,000 per heartbeat, equivalent to 72,000 sperm in a minute, over 100 million in a day, and 3 billion in a month. Furthermore, male gametogenesis (spermatogenesis) can continue for almost the entirety of a man's life. Unlike women, therefore, men can father genetic children at an advanced age even beyond 70 yrs. The foundation of this remarkable functionality of the male reproductive system is spermatogonial stem cells (SSCs), the stem cell population of male germ line. These cells reproduce themselves (self-renew) while continuously generating committed germ cells that are destined to become spermatozoa (commitment/differentiation). This chapter discusses the basic biology of SSCs and their potential for clinical applications.

How are SSCs defined and how many of them are there?

SSCs represent a small fraction of diploid male germ cells, spermatogonia. In general, stem cells are defined functionally by their ability to regenerate and maintain an adult cell lineage. SSCs are thus recognized by their ability to reconstitute spermatogenesis and support continuous sperm production. Such a function is unequivocally detected when cells of interest are transplanted into the seminiferous tubules devoid of spermatogenesis. This technique, called spermatogonial transplantation, was first established in the mouse model. A single-cell suspension of donor cells is prepared by enzymatic digestion of testes and injected into the seminiferous tubules of recipient mice; recipients lack spermatogenesis due to a genetic mutation or a pretreatment with an alkylating agent, such as busulfan (Fig. 1). SSCs included in the donor cell preparation then engraft and regenerate spermatogenesis, and in successful cases, recipients become able to sire offspring through natural mating. Spermatogonial transplantation is thus an unequivocal SSC detection assay based on the functional definition of stem cells. After

transplantation, only stem cells can survive and regenerate spermatogenesis. Non-stem germ cells do not have long-term self-renewal ability and are thus lost through differentiation even if they survive after transplantation. Notably, SSCs have never been purified or made visible under a microscope, emphasizing the importance of spermatogonial transplantation approach to detect SSCs unequivocally. In addition, since a colony of donor-derived spermatogenesis (blue segments seen in Fig. 1) arises from a single SSC, the number of SSCs that engrafted can be determined simply by counting the number of colonies after transplantation. Two unique properties of spermatogonial transplantation are noted. One is that this is an assay that allows for the detection of SSCs 'indirectly' by observing terminally differentiated cells, spermatozoa. The other is that spermatogonial transplantation detects SSCs only retrospectively; we would not know if there are SSCs in a cell sample or how many of them exist in it before transplantation. Using this technique, SSCs have been estimated to represent 0.01–0.02% of total cells in the seminiferous epithelium in mice. SSCs are expected generally to

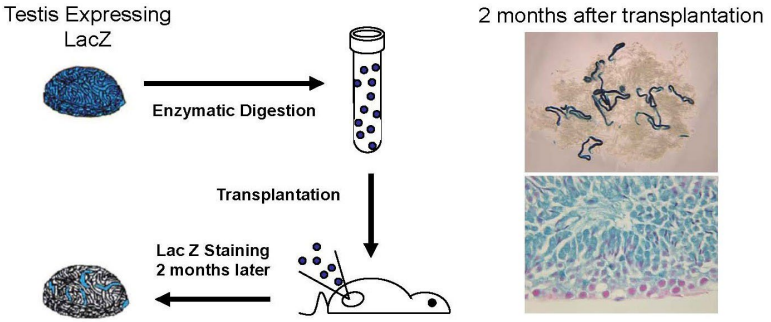

Figure 1. Spermatogonial transplantation in mice. The donor testis is derived from a transgenic mouse carrying the bacterial LacZ gene. The donor testis is digested into single cells using enzymes. These cells are injected into the seminiferous tubules of a recipient mouse that is infertile. Two months later, recipient testes are harvested and reacted with LacZ substrate, which makes donor-derived cells blue. As show 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.

represent a minute subpopulation of spermatogonia in mammalian species.

What is the morphology of a SSC?

Histological analyses of spermatogenesis have contributed greatly to our understanding of male germ cell biology since the 1800's. One unique aspect of male germ cells is that their cytokinesis is incomplete. After each division, male germ cells remain connected by cytoplasmic bridge, like threaded beads, and this morphology of cell chain is maintained until a cohort of spermatozoa are released from the seminiferous epithelium all at once. This also implies that the cells of origin are isolated and not connected with sister cells. These cells are called Type A-single spermatogonia and had long been believed to be stem cells of the male germ line. We now know that A-single spermatogonia are heterogeneous functionally as well as in terms of other characteristics (e.g., genes and proteins expressed); thus, a majority of A-single spermatogonia do not have the regenerative capacity. On the other hand, some studies have also reported that primitive spermatogonia, which are connected with sister cells, may still retain the regenerative activity and act as SSCs; the regenerative capacity has not yet been lost in these seemingly committed cells. The answer to this question about SSC morphology, therefore, must await the purification and reliable visualization of SSCs in the future.

What is the "fate" of SSCs?

After a division of a SSC, two daughter cells emerge, and for these cells, we can consider three potential paths or fate trajectories. If one of the two daughter cells maintains its "stemness" while the other loses the stem cell activity and decides to become spermatozoa, then, SSC self-renewal and commitment to differentiation are balanced, leading to steady-state spermatogenesis (Fig. 2A). If, however, both daughter cells remain as SSCs, this leads to the proliferation of SSCs and an eventual loss of spermatozoa, disrupting spermatogenesis and male fertility (Fig. 2B). SSC proliferation may cause an abnormal expansion of primitive cells that could develop into cancer. On the other hand, a commitment of both daughter cells to sperm formation might lead to a temporal overproduction of spermatozoa and should cause a loss of SSCs, leading to an eventual loss of spermatogenesis (Fig. 2C). This balance of self-renewal vs. commitment needs not to occur at the level

of individual cells; i.e., not every single SSC needs to take the path of balanced cell division. The normal steady-state spermatogenesis can be achieved when the fate is balanced at the population level. For example, a combination of the fate trajectories shown in Figs. 2B and 2C can lead to an optimal balance of inputs and outputs, at least in theory. This thought experiment also tells us that SSCs do self-renew but must not proliferate to maintain normal spermatogenesis and male fertility.

How is SSC fate controlled?

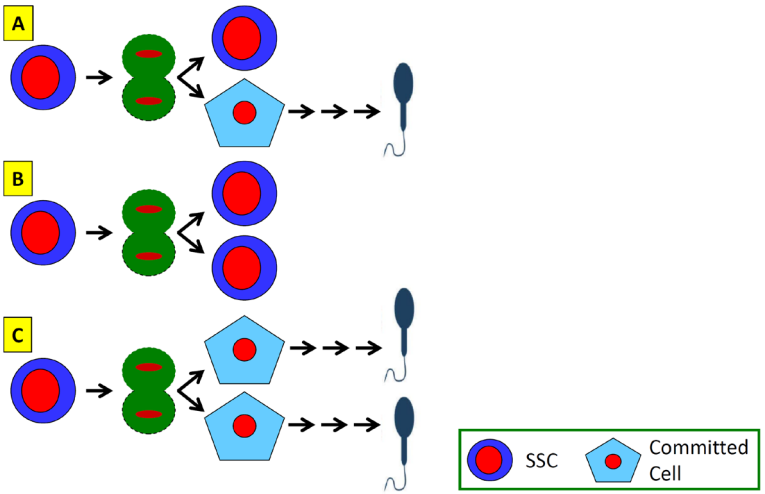

Figure 2. Self-renewal and commitment of SSCs for balanced, steady-state spermatogenesis. SSCs self-renew to maintain a stem cell pool. They can also commit to differentiation to produce sperm. (A) SSC self-renewal and commitment needs to be balanced to maintain normal spermatogenesis. (B) SSCs should not proliferate as a population during normal spermatogenesis. (C) Loss of stemness leads to a temporal overproduction of sperm but eventual loss of spermatogenesis.

This is an area of intensive research and we do not yet know the precise mechanism that controls self-renewal and differentiation of SSCs. However, there are growth factors and hormones that are known to contribute to SSC fate control. Glial-cell-line-derived neurotrophic factor (GDNF) is the critical growth factor that promotes SSC self-renewal. In addition, fibroblast growth factor (FGF) 2 plays

important supportive roles for SSC maintenance. Other growth factors that have been reported to exhibit positive effects on SSC self-renewal include colony-stimulating factor (CSF)-1 and Wnt molecules, whereas retinoic acid is a known differentiation inducer of SSCs and spermatogonia. These factors are secreted by surrounding somatic cells in the environment. The microenvironment that houses stem cells is called a stem cell niche. SSC niches in the seminiferous epithelium are composed of somatic cells, such as Sertoli and myoid cells, acellular elements (extracellular matrices of the basal membrane), and committed germ cells. In addition to soluble factors, SSC niches may influence SSC fate decision through direct cell-cell contact. More studies are required to understand the control mechanism of SSC fate.

Can SSCs be cultured?

Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are stem cells generated in vitro and can be cultured for a long time, leading to exponential propagation. Such a property gives these cells practical advantages in biological research as well as clinical applications since they generate an abundant resource of materials to work with. Despite a far lower efficiency of propagation compared to PSCs, mouse SSCs can also be cultured for a long time and propagated exponentially. Mouse SSC culture requires GDNF and FGF2 as well as a feeder cell layer of embryonic fibroblasts that are replication defective. Another essential component for a successful SSC culture is the use of serum-free and lipid-rich medium. Under these conditions and through serial passaging over time, mouse SSCs can be propagated in vitro. Of note, however, is that a majority of cultured spermatogonia are not SSCs, even though they respond to GDNF and FGF2; it is estimated that only ~1–3% of cultured spermatogonia have regenerative capacity. Studies have reported that mouse SSCs are stable in their karyotype and epigenetic marks (e.g., DNA methylation) during long-term culture, in contrast to ESCs, which readily acquire epigenetic and karyotypic abnormalities in vitro. Rat SSCs can also be cultured similarly as mouse SSCs but are far more difficult to maintain in vitro. In vitro culture and amplification of SSCs in other animal species have been attempted and reported but consistent success has been hard to be achieved.

What are the potential practical/clinical applications of SSCs?

Being the foundation of spermatogenesis, SSCs are expected to become a critical resource for male fertility preservation and restoration. For example, anti-cancer therapies can induce male infertility. While sperm cryopreservation is an important option for adult men, it is not applicable for prepubertal boys due to their developmental immaturity. However, since SSCs exist in the testis from the time of birth, these cells can be harvested before the therapy, cryopreserved, and transplanted afterwards to regenerate spermatogenesis. Fertility may be restored spontaneously or via assisted reproductive technologies. Similarly, SSCs of non-human animal species can be used for preservation of species or favored agricultural traits. SSCs can also be used for germline gene modifications. In fact, gene editing in SSCs and even transchromosomal transgenesis via SSCs have been reported in mice to modify the phenotype of offspring. It is important to note that every single step of the SSC-based strategy of male fertility preservation and restoration (harvest testis cells including SSCs, enrich the cells for SSCs, cryopreserve SSCs, propagate SSC in vitro, transplantation, mating that results in offspring production) has been realized in the mouse model. The efforts to translate this achievement in clinical settings are currently underway world-wide.

What is the male mutation bias for transmission of genetic diseases and its linkage to SSCs?

There are inherited diseases and familial syndromes caused by genetic mutations that are transmitted to children solely by fathers, the phenomena called the male mutation bias. Interestingly, the frequency of such diseases increases as fathers become older. A particularly strong male mutation bias has been reported in point mutations of Ret, which is a signal-transducing component of the GDNF receptor, as well as FGF receptor (FGFR) 1 to 3. These mutations result in constitutive activation of these receptors and their down-stream signaling cascades. Ret mutations lead to multiple endocrine neoplasia 2A and 2B and familial medullary thyroid carcinoma while FGFR mutations cause Apert, Crouzon, and Pfeiffer syndromes. Interestingly, GDNF and FGF are two essential growth factors required for long-term amplification of mouse SSCs in vitro (see above). It has thus been proposed that the constitutive

activation of signaling pathways that are beneficial for propagation of SSCs and primitive spermatogonia gradually give these cells proliferative advantage over time, eventually taking over a significant proportion of the sperm production, which results in male mutation biases that become more prevalent as a father ages.

What are SSC markers?

Similar to most bioassays, the functional detection of SSCs provides the information only retrospectively, which makes SSC research difficult and data interpretation confusing. Prospective detection of SSCs requires "SSC markers". A number of marker molecules have been identified at the transcript and protein levels for SSCs as well as their descendants. If such markers are cell-surface proteins, cells expressing and not expressing a marker or markers can be sorted and harvested using immunological methods, such as fluorescent-activated cell sorting or immunomagnetic cell separation. When transplanted, if marker-expressing cells generate more colonies of donor-derived spermatogenesis than unsorted control cells, then, the marker protein is called a positive SSC marker. If colony numbers decline, such a marker is considered to be a negative SSC marker. Immunological cell sorting using these proteins can increase the concentration of SSCs (SSC enrichment), but SSCs have not been purified even in the mouse model and cannot be visualized unequivocally. Considering multiple markers are required to identify hematopoietic stem cells (or a cell population that is highly enriched for these stem cells) in mice and humans, it is logical to assume that SSC identification may also require multiple markers. Thus, it is necessary to be cautious in interpreting data when SSCs are defined or analyzed only by a single gene or protein. Probably, the most important issue regarding SSC markers, which calls for particular attention and care, is the fact that gene X or protein Y may be expressed by SSCs, but not all the cells that express such a gene or a protein are SSCs. For example, GFRα1 is a receptor of GDNF and is a SSC maker, but it is also expressed by primitive spermatogonia that have lost regenerative activity; thus, not 100% of GFRα1-expressing cells are SSCs.

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Chapter 9
What makes the process of spermatogenesis unique?

Stefan Schlatt and Swati Sharma

The generation of gametes is relevant for any higher species since recombination during meiosis is a crucial event establishing genetic diversity. The primary task of the gonad is homing of primordial germ cells, guidance of germ cells towards a species- and sex-dependent differentiation, adequate expansion of germline stem cells and meiosis and postmeiotic germ cell differentiation. The process of spermatogenesis is the unique male-specific track of germ cell development leading to the generation of sperm.

Unique somatic differentiation of the testis (red and orange box)

The initially indifferent gonadal anlage undergoes sex-specific gene expression during embryogenesis. The gene SRY on the Y-chromosome kicks on a cascade of gene expression leading to the appearance of a first testis specific cell type called Sertoli cells. These cells form dense aggregates engulfing primordial germ cells. The aggregates reorganize to develop into seminiferous cords which grow out longitudinally to form the seminiferous tubules making up 90% of the adult testicular mass. Newly formed cords terminate at the rete testis. Products are released to the outside via the initial segment (mesonephros derivative), efferent ducts and the epididymal and spermatic ducts. The seminiferous cords are lined by a basement membrane with the seminiferous epithelium populating the inside and peritubular cells colonizing the outside of the cords. The testis becomes a bicompartmental (and bifunctional) organ separated into a tubular (spermatogenesis) and an interstitial (steroidogenesis) compartment. Sertoli cells are the principle components of the seminiferous epithelium with tight basolateral contacts functioning as the blood-testis barrier. Leydig cells are testis-specific cells in the interstitium producing primarily androgens.

Initial male germ cell development (yellow boxes)

Primordial germ cells populate the indifferent embryonal gonad. In the male gonadal anlage, they are engulfed by Sertoli cells and are now considered gonocytes which remain mitotically quiescent. Later during development, gonocytes settle on the basement membrane between Sertoli cells to form a population of A-spermatogonia which function as reserve stem cells for the life-long generation of spermatozoa (Chapter 8).

Premeiotic spermatogonial expansion (red box)

The pool of A-spermatogonia consists of several subpopulations constituting a unique stem cell system. Features of the stem cell system differ between species. In general, stem spermatogonia rarely divide to give rise to cyclically proliferating A-spermatogonial populations. Individual A-spermatogonia are mobile cells migrating along the base of the seminiferous tubules. The dividing spermatogonia rarely complete mitosis and usually form interconnected cell clones. Depending on the species, few to several mitotic divisions culminate in spermatogonial clones of different size (primate: 32-64 cells, rodents: several hundred to thousand cells). Clones may eventually split into single cells or duplets to remain stem cells or as more extensive clones, as they enter the process of spermatogenic differentiation.

Meiosis (green box)

Meiosis is the most relevant process during spermatogenesis providing a track for haploidization with intense recombination of the genome (Chapter 10). The germ cell clones enter prophase of meiosis after a last S-phase prior to the preleptotene stage. The entry into meiotic prophase initiates a highly synchronized developmental program lasting for several weeks while cells pass through prophase of meiosis and subsequently undergo the two meiotic divisions. When entering meiosis germ cell clones disconnect from the basement membrane and become embedded between Sertoli cells. The blood testis barrier is reestablished basally before it opens apically keeping a tight blood testis barrier in the seminiferous epithelium.

Germline checkpoints assuring genomic integrity

The exquisite sensitivity of spermatogonia to toxins or radiation indicates unique checkpoint mechanisms. Even lowest doses of toxicant exposure or radiation impact and/or deplete all differentiating spermatogonia. Resting stem cells are less sensitive to damage and depletion. Depending on the intensity of exposure, they remain in the seminiferous epithelium. Restoration of spermatogenesis occurs via activation of the dormant stem cell populations. Repopulation from stem cells initiates either full, partial or no restoration of spermatogenesis. The exact target cell subpopulations and selective mechanisms for quality control checks in premeiotic germ cells remain to be established; however, the existence of rigorous selection among male germ cells is a unique feature of spermatogenesis.

Limits of clonal spermatogonial expansion (blue box)

Another unique feature of mammalian spermatogenesis is a peculiar cellular and histological organization. The seminiferous epithelium contains several layers of germ cells. Expanding A-spermatogonia form flattened interconnected clones along the basement membrane. All subsequent mitotic and meiotic divisions are highly synchronized enabling to define stages of the seminiferous epithelial cycle by morphological criteria visible in the synchronized cohorts of germ cells. Among other criteria, the acrosome development in round spermatids is a primary marker for definition of seminiferous epithelial stages. Species showing large germ cell clones due to extensive premeiotic expansion show a longitudinal arrangement of seminiferous epithelial stages (e.g. rodents) whereas species with small clones (e.g. primates) reveal mixed arrangements of seminiferous epithelial stages in individual tubular crossections.

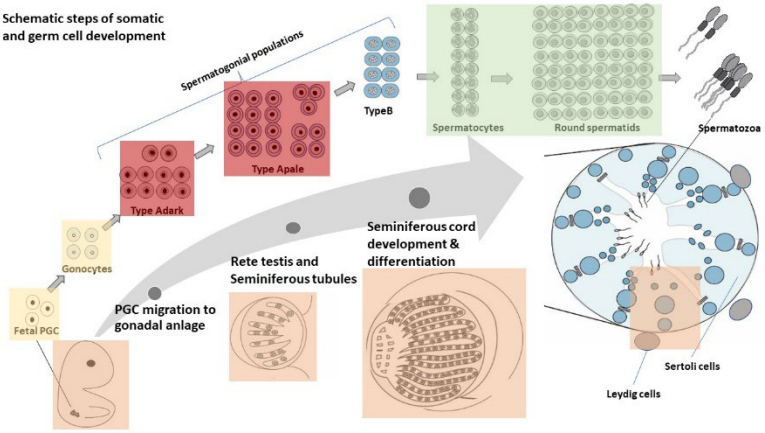

Figure 1. Schematic steps of somatic and germ cell development

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Chapter 10
How do sperm get the correct number of chromosomes?

Mary Ann Handel

Germ cells use meiosis to ensure gametic haploidy. Meiosis is a unique and defining event of gametogenesis serving at least two functions in the reproductive life cycle: it reduces chromosome number to a perfect 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 and chromosomal content to haploidy. The success of these divisions depends on the unique dynamics of chromosomes during the extended meiotic prophase in primary spermatocytes.

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, and 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, chromosomes pair and homologs

initiate their intimate association 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 can be 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 chromosomes 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, maternal homolog oriented to one pole and paternal homolog to the other. 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. As is the case with 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.

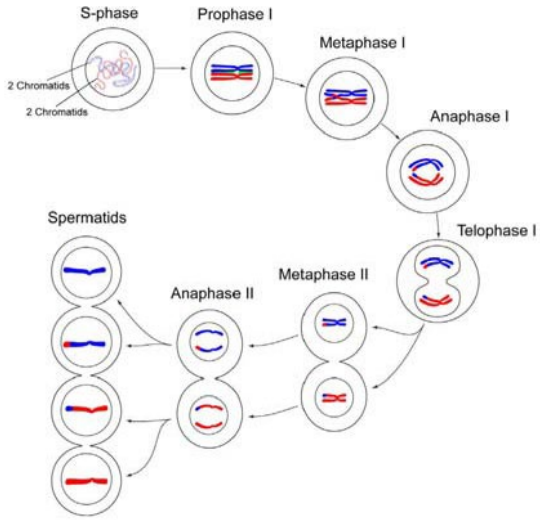

Figure 1. This diagram illustrates the sequence of key events of meiosis in male germ cells. Red and blue depict the two homologs of an autosomal chromosome, one maternally derived and the other paternally derived. At the completion of S-phase, each chromosome consists of two chromatids. At the pachytene stage of prophase I, homologs are synapsed to form a bivalent, an event mediated by the synaptonemal complex (green, seen in Prophase I). 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.

What do we know about molecular regulation of these remarkable and precise chromosomal dynamics, how do we know it, and is this information useful in the clinic? We know that the processes of creating and repairing DNA double-strand breaks (DSBs) drive much of the chromosome interactions during meiotic prophase. Mutations impairing function of any of the many DSB-

related proteins cause arrest of male germ-cell development in meiotic prophase with subsequent infertility. Much of this information has derived from programs exploiting deliberate chemical mutagenesis or CRISPR gene targeting of the mouse genome. These endeavors have identified a plethora of genes required for spermatogenesis, many of which control meiosis, a seemingly particularly vulnerable stage. More recently, single-cell RNA sequencing is identifying the transcriptomes of cells throughout spermatogenesis and this too is leading to the discovery of genes that control the progress and tempo of meiosis, ensure gametic haploidy, and lay the foundation for post-meiotic spermiogenic differentiation.

With the identification of molecular mechanisms of meiosis, much attention has been directed to how the meiotic choreography of chromosome movement can go wrong, and whether these errors can explain any cases of human infertility. The penalty of meiotic error can be germ-cell arrest or generation of aneuploid gametes and offspring bearing the wrong number of chromosomes. Either 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, where it is prevalent and age-related. It appears that in males meiotic errors are detected and defective germ cells are eliminated. Infertility due to arrested spermatogenesis and germ cell death ("maturation arrest") is found in some human male infertilities. 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 has been difficult to establish. Indeed, it is even the case that when modeled in mice, some human mutations predicted to be deleterious do not cause meiotic arrest or infertility. Interestingly, some mouse meiotic mutations that cause germ-cell arrest and infertility on one genetic background do not on other genetic backgrounds. Lines of evidence such as this strongly suggest considerable inter-individual

genetic diversity in the phenotypic expression of mutations affecting meiosis and other aspects of spermatogenesis, a cautionary note warning against over interpretations of male meiotic arrest infertility in the clinic.

In a summary of the essentials, meiosis is a defining event of spermatogenesis and is comprised of two divisions (Fig. 1). Driving progress of meiosis are the events of synapsis of homologous chromosomes and reciprocal recombination, resulting in new combinations of gene alleles. The first reductional division separates homologous chromosomes and reduces chromosome number from 2N to 1N. The second division is equational, separating chromatids. The products of these meiotic divisions are spermatids, each now with the genetically correct haploid 1N chromosome number and 1C DNA content.

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Chapter 11
What are the roles of androgens and FSH in the hormonal regulation of sperm production?

Robert I. McLachlan and Liza O'Donnell

Overview

Spermatogenesis is the process of sperm development and features mitotic and meiotic divisions, elaborate cytodifferentiation and changing inter- cellular interactions. Sperm are produced within the seminiferous tubules from puberty onward, and spermatogenesis is supported by the somatic Sertoli cells which provide structural and nutritional support. Sperm production is driven by the interplay of autocrine, paracrine and endocrine factors. Endocrine systems regulate both the initiation (neonatal development and puberty) and maintenance of spermatogenesis via a classic negative feedback system (Fig. 1) involving elements of the hypothalamic-pituitary-testis (HPT) axis. Sperm production is dependent on the pituitary gonadotropins, LH and FSH, that are secreted in response to hypothalamic gonadotropin-releasing hormone (GnRH). LH acts on LH receptors (LH-R) in Leydig cells to simulate steroidogenesis and testosterone secretion. Testosterone acts on androgen receptors (AR) in the testicular somatic cells to stimulate sperm production, and also feeds back on the hypothalamus and pituitary to regulate GnRH secretion. FSH acts on receptors (FSH-R) within Sertoli cells to support optimal spermatogenesis, and to produce inhibin B that has a negative feedback effect on pituitary FSH secretion. Interestingly, LH-R and FSH-R share the same chromosomal location, suggesting a common ancestry. The physiology of these key endocrine regulators of spermatogenesis are outlined here and are illustrated by approaches to hormonal contraception and the treatment of infertility.

Androgen action and the androgen receptor (AR)

Androgens are essential for the initiation and maintenance of spermatogenesis (Chapter 3). Androgens act on AR in somatic cells to support spermatogenesis, but germ cells lack AR. Many androgen actions on germ cell development are thought to be mediated via AR in Sertoli cells; however, an action on AR within the peritubular myoid cells surrounding the tubules (Fig. 1) is also required for normal sperm production. In mice lacking AR in Sertoli cells, spermatogenesis does not proceed beyond the early meiotic prophase, indicating that the completion of meiosis depends on androgen action. Reduced androgen levels in the testis are associated with defective meiosis, spermiogenesis, germ cell survival and reduced sperm release, highlighting that androgen action supports multiple aspects of spermatogenesis. Androgen action promotes the pubertal maturation of Sertoli cells and maintains specialized tight junctions between Sertoli cells (Fig. 1) that are necessary for meiotic and post-meiotic germ cell development. Androgen action via AR is also important for the development and function of the adult Leydig cell population which, in turn, is required for normal testosterone synthesis.

Testosterone, or its 5α-reduced metabolite dihydrotestosterone (DHT), binds to cytosolic AR which then dimerize, 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 or "classic" pathway of action. Androgens can also elicit responses via the non-genomic or "non-classic" pathway where AR bound ligand migrates to the plasma membrane to initiate specific intracellular signalling pathways. Studies in mice indicate that both pathways co-operate, and are required in Sertoli cells to support normal spermatogenesis.

FSH and its Receptor (FSH-R)

FSH-R is expressed only in Sertoli cells. FSH action on this receptor initiates signal transduction events including cAMP stimulation and Ca^{++} release leading to intracellular signalling cascades, such as protein kinase-A and C dependent pathways. Studies in mice show that FSH is particularly important for the proliferation and development of the adult Sertoli cell population which in turn determines optimal sperm output. Although sperm production can occur without FSH action in mice, rats and perhaps man, optimal germ cell survival and the production of normal sperm numbers requires FSH action.

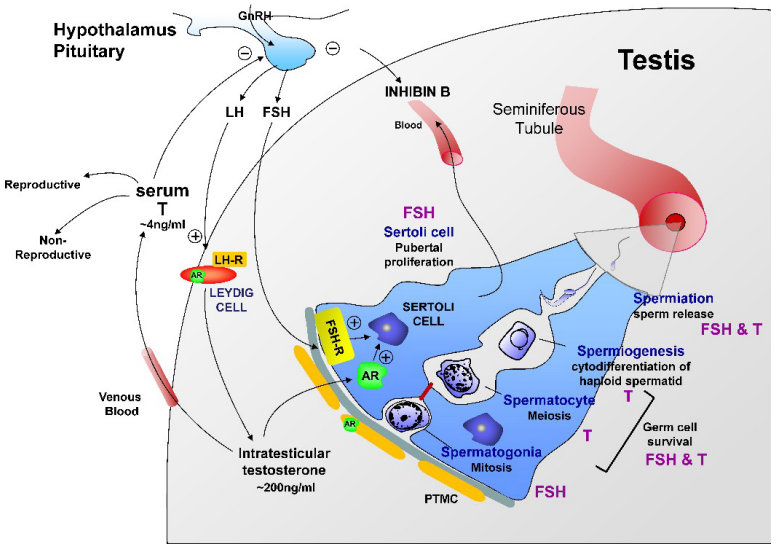

Figure 1. The regulation of spermatogenesis by androgen and FSH. FSH; follicle stimulating hormone, LH; luteinizing hormone, GnRH; gonadotropin-releasing hormone, LH-R; LH receptor, FSH-R; FSH receptor, AR; androgen receptor, PTMC; peritubular myoid cells. Note the location of FSH- R and AR in Sertoli cells that transduce these effects to complete spermatogenesis, and the expression of AR in Leydig cells and PTMCs. Inter-Sertoli cell junctions are denoted by the red line. 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 to 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.

The dependence of human spermatogenesis on androgen and FSH

FSH and testosterone support the initiation of spermatogenesis and both are needed for quantitatively normal spermatogenesis in men. The requirement for these hormones in human spermatogenesis is the physiological principle behind male hormonal contraception

wherein exogenous testosterone (with or without progestin) administration reduces pituitary LH and FSH secretion rendering the majority of men (reversibly) azoospermic. This requirement is also the basis of combined gonadotropin therapy to restore fertility in hypogonadotropic hypogonadism (HH) that may result from a deficiency of hypothalamic GnRH drive or pituitary gonadotropin secretion (see Fig. 1). In congenital HH where the testes have not been exposed to FSH or androgens, germ cells do not proceed beyond the spermatogonial stage. In HH acquired after puberty (where pubertal testis development is normal), the testes exhibit spermatogenic regression and markedly reduced sperm counts (oligo/azoospermia). In situations of partial HH, reduced sperm output may permit fertility but complete HH is associated with the cessation of spermatogenesis and infertility.

Testicular androgen physiology

Both the initiation of spermatogenesis at puberty, and its maintenance during adulthood, requires androgen action. Due to its production by Leydig cells in the testicular interstitium (Fig. 1), testosterone is present in high concentration in the testis (>50 fold that in serum). In adults, spermatogenesis can proceed when testicular testosterone levels are lower than normal, but the initiation of pubertal spermatogenesis requires higher levels of androgen support.

Interesting observations regarding androgen regulation of human testicular function include:

- In men engaging in performance-enhancing androgen abuse, serum androgen levels are greatly elevated and spermatogenesis is severely regressed in line with gonadotropin suppression; thus even very high serum levels of exogenous androgens are not able to maintain spermatogenesis.
- The ability of androgens alone to initiate human spermatogenesis is evidenced by the appearance of seminiferous tubules with germ cell differentiation adjacent to androgen secreting Leydig cell tumors in pre- pubertal boys
- During testosterone (±progestin) administration for the purpose of hormonal contraception, LH levels are profoundly suppressed, yet residual intra- testicular testosterone (iTT) levels remain ~ two-fold higher than normal adult serum levels (Chapter 30). While most men given this treatment become azoospermic or severely oligospermic, ~5% of men demonstrate that spermatogenesis is not adequately suppressed for contraceptive efficacy.

Some men may exhibit higher levels of the enzyme that converts testosterone to the more potent androgen dihydrotestosterone, which could preserve some level of androgen activity in the testis. However, the reason why some men fail to achieve adequate sperm count suppression remains unknown.

- A fundamental question arises as to the mechanism of androgen action within the testis: how does one understand the relationship between androgen levels and AR receptor function given that the dissociation constant of the testicular AR is 3 x 10-9M suggesting it is always fully saturated by the very high levels of androgen in the normal testis? What co-factors modulate androgen action in this unique setting? Why are the levels adequate for the full expression of androgen action in non-gonadal tissues so clearly inadequate for spermatogenesis?

The role of FSH in human spermatogenesis

Based on studies in rodents, it is likely that a major, independent role of FSH in establishing the spermatogenic potential of the testis is by promoting Sertoli cell proliferation and development of the functionally competent, adult Sertoli cell population (Chapter 3). This is evident in human spermatogenesis by the fact that FSH administration is frequently required to establish spermatogenesis in congenital HH. Also, the time to spermatogenic induction is shorter in the second round of treatment after a successful first course, pointing to a permanent effect of FSH on Sertoli cell maturation necessary for spermatogenesis. In rodents, spermatogonial development is particularly reliant on FSH, but in humans this requirement is less well understood.

Case reports of rare men with mutations in the FSH gene (FSHβ) or the FSH-R reveal some inconsistencies in the spermatogenic phenotypes: men with FSH-R mutations have moderately elevated FSH levels and variably severe reductions in sperm counts, whereas men with an inactivating FSHβ mutation are azoospermic. This latter observation contrasts with data in mice lacking FSHβ where fertility is preserved. This observation suggests that human spermatogenesis may be more reliant on FSH to establish / maintain spermatogenesis.

Co-operation between androgen and FSH is required for normal spermatogenesis

There is abundant evidence that both hormones are required for quantitatively normal spermatogenesis and that they co-operate, particularly within Sertoli cells, to support multiple aspects of Sertoli and germ cell development. Spermatogenesis can proceed without FSH (at least in rodents), but due to its role in supporting peri-pubertal Sertoli cell proliferation and germ cell survival, the testes are smaller and sperm count is reduced in the absence of FSH.

The requirement for androgens and FSH in human spermatogenesis is evidenced by studies in men undergoing hormonal contraception (exogenous testosterone ± progestin therapy). Contraception-induced gonadotropin suppression in normal men inhibits spermatogenesis, due to an inhibition of spermatogonial maturation, accelerated germ cell apoptosis during meiosis and spermiogenesis, and the failure of sperm to be released by Sertoli cells at the end of spermatogenesis. Studies in men show that either LH or FSH can support these aspects of spermatogenesis during gonadotropin suppression, highlighting their ability to co-operate.

Androgens and FSH have individual, overlapping and synergistic effects on spermatogenesis, and the threshold of action of one hormone is lower when the other hormone is present. For example, a patient with a mutated form of LH exhibited very low testosterone production, very few Leydig cells, but high FSH and the presence of spermatogenesis in his testes, highlighting the remarkable co-operation between androgens and FSH in supporting fertility. In men with experimental gonadotropin suppression, sperm production was restored to 50% of baseline values by either FSH or human chorionic gonadotropin [hCG] (as an LH substitute), whereas only combined hCG plus FSH treatment led to quantitative restoration.

In men with HH, the restoration of endogenous gonadotropin secretion or exogenous gonadotropin replacement therapy (usually using hCG as an LH substitute and human FSH) will induce/restore spermatogenesis in most cases; however, this may take many months given that it takes more than two months for human spermatogonia to develop into spermatozoa. In the case of severe congenital HH, where the Sertoli cell population has not been able to mature, several years of gonadotropin therapy may be required to stimulate fertility.

Conclusion

Androgen and FSH act on receptors within the testes to support somatic cell function and to stimulate spermatogenesis. Androgen action is essential for the production of sperm, and FSH action is required for the development of the functionally normal Sertoli cell population during puberty. Both hormones co-operate and synergize to ensure optimal spermatogenesis and fertility.

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Chapter 12
Can sperm be produced *in vitro*?

Heather Steele and Ina Dobrinski

Spermatogenesis, the production of male gametes, is initiated at puberty and through the self-renewal and differentiation of spermatogonial stem cells (SSCs) enables lifelong male fertility. The testicular microenvironment is critical to this delicate balance, as SSC maintenance and differentiation is controlled by various factors within the somatic niche. The SSC population is extremely limited within the testis and these cells are particularly susceptible to gonadotoxic agents such as radio- and chemotherapy. This means that the SSC pool may be decimated during cancer treatment, rendering patients infertile. As such, fertility preservation should be undertaken prior to treatment. For adult men this is a relatively simple process as semen samples can be collected and cryopreserved for future use with assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Chapter 35). Semen cryopreservation is reliable and inexpensive. However, fertility preservation of prepubertal boys poses a challenge (Chapter 24). How can we preserve their fertility when they do not yet produce sperm? The answer may lie at the bottom of the SSC pool.

One alternative male fertility preservation strategy could be the cryopreservation of testicular biopsies, taken prior to treatment, as whole tissue or as testicular cells isolated after enzymatic digestion of the testis biopsy. These samples can be stored until patients reach adulthood. However, testicular biopsies require much greater *in vitro* intervention than processing cryopreserved sperm for ARTs. As the testicular tissue is removed from a patient diagnosed with cancer, it is inadvisable to re-transplant that tissue later in adulthood due to the risk of reintroducing malignant cells harboured in the biopsied testicular tissue. This necessitates the need for *in vitro* culture methods to propagate SSCs and to produce haploid male gametes (Fig. 1). As of yet, this has not been achieved in species other than rodents and so is not practicable for therapeutic use. Thus, the recapitulation of spermatogenesis *in vitro* represents a major challenge in reproductive medicine of the 21st

century and is essential in achieving functional fertility preservation for prepubertal boys.

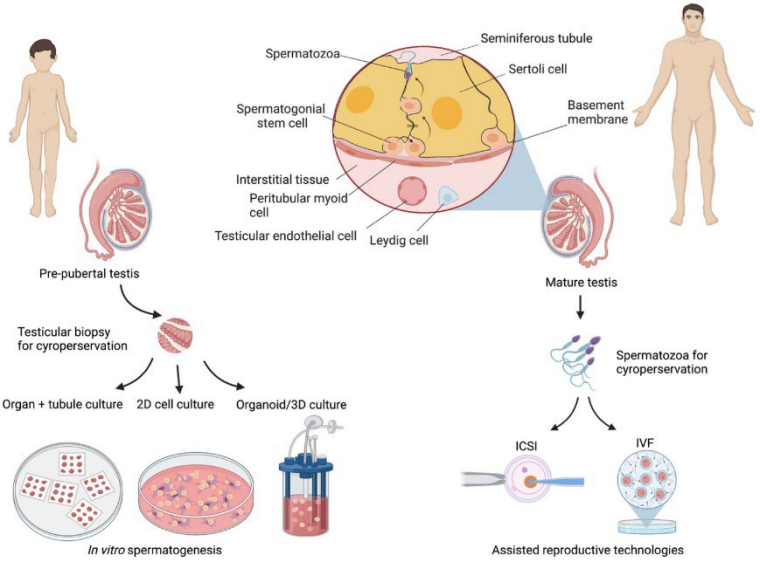

Figure 1. Schematic of the fertility preservation process for prepubertal boys and sexually mature men. The left side of the figure shows the *in vitro* methods that have been developed in a bid to recapitulate spermatogenesis *in vitro*. The right side shows the relatively simple fertility preservation process of sexually mature men, who can cryopreserve spermatozoa for later use in assisted reproductive technologies. If *in vitro* spermatogenesis is successful, the produced spermatozoa would also require assisted reproductive technologies in order to produce a viable embryo.

The effort to produce sperm *in vitro*, spanning over a century, aims to recapitulate the niche environment to initiate and sustain the successive steps of spermatogenesis through mitosis and meiosis to produce haploid male germ cells. The purpose of *in vitro* spermatogenesis (IVS) is two-fold; to enable the production of fertile sperm from biopsied tissue from patients with impaired spermatogenesis and to better understand the basic biology of spermatogenesis. In pursuit of IVS researchers have tried various

methods including different culture strategies, media supplementation and working within a variety of species. Nonetheless, the recapitulation of spermatogenesis *in vitro* has remained challenging.

Primarily three methods have been used for IVS including organ/tubule culture, cell culture and 3-D/organoid culture. Organ culture, using tissue pieces *in vitro*, is the longest established IVS method dating back to the 1920s. Here the first reports were made of *in vitro* differentiation of germ cells using pieces of rabbit testis cultured in rabbit plasma allowing the survival of undifferentiated germ cells and somatic cells for a brief period. Organ culture is particularly useful as germ cells remain spatially arranged as they are in vivo, somatic cells are maintained and cells are protected from the deleterious effects of enzymatic digestion. In the 1960s, organ culture was improved with the development of the gas-liquid interphase method, allowing tissue fragments to survive for longer periods facilitating spermatogenesis from zygotene to the pachytene stage. Additionally, developments in electron microscopy and flow cytometry enabled more precise discrimination of stage specific spermatogonia. However, a key challenge of this era was the support and maintenance of tissue viability which curtailed long term culture in organ culture systems.

In a bid to avoid whole testicular fragments, researchers in the 1980s focused their attention to 2D cell culture of enzymatically digested testicular cells. Dissociated testicular cells are of particular value in studying the role that different niche factors play in germ cell differentiation as the challenge of tissue degradation is avoided. Much of cell culture within IVS has relied on the use of feeder cell populations to sustain SSCs. Many different cell types have been trialled as feeder cells with OP9 bp stroma or fibroblastic cells having been identified as best suited to supporting mouse germ cell maintenance. Porcine Sertoli cells have been suggested as critical feeder cells for human spermatids, limiting apoptosis. This inter-species variation in response to different feeders highlights the challenge in translating these systems. This is further illustrated by the failure to translate results from the first culture system to support the growth and proliferation of murine spermatogonia to other species. This system used mouse fibroblasts as feeder cells supplemented with GNDF (glial cell line derived neurotrophic factor), FGF2 (fibroblast growth factor 2), EGF (epidermal growth factor) and LIF (leukaemia inhibitory factor). However, these conditions did not support SSC maintenance from larger non-rodent animals and humans. This translational barrier may be due to

differences in the metabolic requirements during the extended prepubertal stage of larger mammals compared to rodents.

However, it was by returning to organ culture that *in vitro* spermatogenesis succeeded. In a landmark paper by Sato et al., 2011, *in vitro* production of functional sperm in cultured neonatal mouse testes was finally achieved. Using neonatal mouse testis containing gonocytes or primitive spermatogonia, spermatids and sperm were produced *in vitro* using serum free media. This system maintained spermatogenesis for over two months in tissue fragments at the gas liquid interphase and produced healthy offspring via ICSI. This has since been replicated in rodents by a number of groups; however, successful culture in non-rodent species has remained intractable.

The modern era has seen the adoption of a number of new IVS systems in an attempt to develop a more translatable system. Microfluidic technology has been integrated into organ culture to replicate the circulatory system of the body. In this adapted system, cultured testis tissue is separated from flowing medium by a thin, porous membrane to allow for nutrient and waste exchange via diffusion mimicking the in vivo niche. This novel organ culture system facilitated testosterone production, which was supported by addition of luteinizing hormone, and maintained spermatogenesis in mouse samples for more than 6 months producing healthy offspring via ICSI. The most recently developed IVS system is 3D/organoid culture. This system has advantages over previous static 2D culture systems as the SSCs and somatic cells may self-organise to reassemble the microarchitecture of the niche and allow greater cell interaction. The first 3D system was designed in 2006 and used testicular cells from juvenile rats cultured on collagen gels to mimic the basal membrane and supported limited germ cell differentiation. Recent developments have seen researchers utilise testicular cells in microwells to form aggregates and in turn organoids consisting of germ cells, Sertoli cells, Leydig cells, peritubular myoid cells with distinct seminiferous epithelium and interstitial compartment separated by a basal membrane. Importantly these organoids have been developed in a number of larger mammals including pigs, macaques, and humans which in the future may facilitate sustained and successive SSC differentiation.

Nonetheless, despite a century of research investigating the recapitulation of spermatogenesis *in vitro*, much remains unknown. In order to develop suitable culture conditions, the repertoire of signalling factors required by SSCs including growth factors,

hormones, signals, and interactions between SSCs and the constituent somatic cells of the niche must be fully elucidated. Spermatogenesis is a highly regulated process occurring over a long period of time requiring orchestrated events from germ and somatic cells within the testes composed of 3 distinct phases – proliferation of spermatogonia, meiotic division of spermatocytes, distinct changes in shape and nuclear contents of haploid spermatids (spermiogenesis). Several of these factors required for SSC self-renewal have been identified. Sertoli cells secrete GDNF and FGF2, while CSF1 (colony stimulating factor 1) is secreted by the Leydig and peritubular myoid cells. However, these do not represent the entirety of the signalling molecules regulating the SSC niche microenvironment, and it is highly likely that a number of additional niche factors such as specific miRNAs remain to be identified. Another challenge is the lack of a definitive marker for SSCs. To achieve sufficient numbers of cells required for effective experimentation and therapy development, the spermatogonial population, which contains SSCs, must be increased *in vitro*. Finally, each species has a unique SSC niche which poses a challenge in translating murine culture systems. The full complement of signalling factors within more translational models such as the pig and primates must be uncovered in order to develop more attuned culture systems that may support human *in vitro* spermatogenesis.

At present, to answer the question whether sperm can be produced *in vitro* depends on which species you are interested in. The successful recapitulation of spermatogenesis in rodent species is a significant achievement towards this goal; however, to do so in non-rodent models, especially humans, remains challenging. Recent developments in organotypic culture coupled with the continuing identification of the full repertoire of signalling factors within the niche, as well as further refinements to media supplementation may allow *in vitro* spermatogenesis from larger animals in the near future. Achieving *in vitro* spermatogenesis in non-rodent species is the next hurdle in developing functional fertility restoration for prepubertal patients, enabling pharmacological and toxicological study of new drugs on the testis and the genetic manipulation of germ cells which is not possible *in vivo*.

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Chapter 13
Do DNA methylation patterns change during spermatogenesis? What is the role of imprinting of male germ cells?

Gurbet Karahan and Jacquetta M. Trasler

Epigenetics refers to heritable mechanisms of modulating gene expression that do not involve alterations in DNA sequence. DNA methylation, histone modifications and small non-coding RNAs are the three main types of molecular information 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 diseases such as cancer and imprinting disorders.

DNA methylation is the most extensively characterized epigenetic mechanism. DNA methylation is found most frequently 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 70-80% of cytosines within CpGs are methylated. The functions of DNA methylation include roles in the transcriptional repression of retrotransposons and single copy genes (at their CpG-rich promoters), the differential 'marking' of imprinted genes to ensure parent-of-origin specific expression, X chromosome inactivation and modulating the access of transcription factors to gene regulatory regions.

Two types of DNA methylation occur: i) *de novo* methylation or the acquisition of methylation on unmethylated cytosines, or ii) maintenance methylation, which takes place at the time of DNA replication to ensure the propagation of DNA 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 differentially methylated regions (DMRs) of imprinted genes. Methylation of DNA is catalyzed by a family of DNA (cytosine-5)-methyltransferases (DNMT enzymes or DNMTs). DNMT3A and DNMT3B are the main DNMTs involved in the establishment (*de novo* methylation) of DNA methylation patterns whereas DNMT1 plays the

key role in DNA methylation maintenance. Although it lacks enzymatic activity, DNMT3L (DNMT3-like) is related to and works with DNMT3A and DNMT3B. A recently identified rodent-specific DNMT, DNMT3C, has been shown to methylate only a very specific subset of sequences in the genome, young transposable elements (TEs) in the male germ line. DNA methylation can be erased by either passive or active demethylation mechanisms. Passive DNA demethylation is achieved by replication coupled dilution of methylated DNA and sequestration of the maintenance and *de novo* DNA methylation machinery. Ten-eleven translocation (TET) methylcytosine dioxygenases (TET1, TET2 and TET3 enzymes) actively demethylate DNA.

DNA methylation patterns are very dynamic during embryonic development and the establishment of the germ cell lineages (Fig. 1). Following fertilization, the paternal genome goes through a rapid erasure involving active and passive demethylation. In contrast, the maternal genome loses DNA methylation by passive demethylation, more gradually during preimplantation development. Imprinted gene DMRs and some other loci are resistant to this global wave of demethylation. Imprinted gene DMR methylation is maintained during preimplantation development since it is only in the germline (male or female depending on the gene) that a subset of imprinted genes acquire the allele-specific methylation that will result in monoallelic expression in the embryo and postnatal individual.

After implantation, lineage-specific DNA methylation patterns are established. Precursors of eggs or sperm (primordial germ cells – PGCs) go through another round of demethylation to reset marks coming from the parents, to reach a genomic average of <10% DNA methylation, the lowest level of CpG methylation occurring in the genome of a cell (Fig. 1). This erasure is particularly important for imprinted gene DMRs as maternal 'marks' on imprinted genes inherited from the mother must be erased and new paternal 'marks' introduced. Although erasure of DNA methylation at the PGC stage is the most extensive DNA demethylation to occur in any cell, not all of the genome is demethylated. A subset of sequences such as young retrotransposons (marked by a histone modification – H3K9me3) is resistant to demethylation in PGCs.

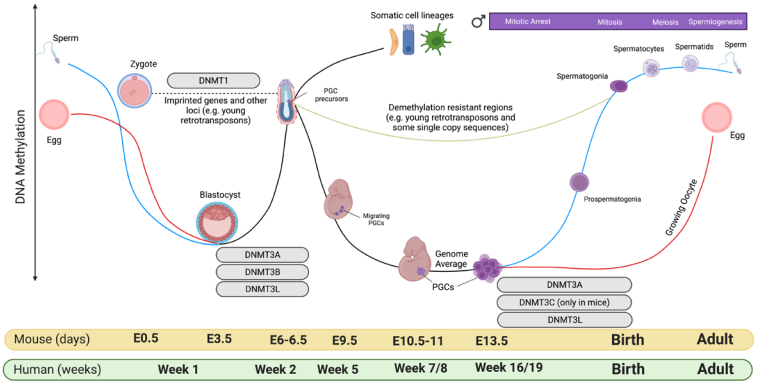

Figure 1: Epigenetic reprogramming of DNA methylation patterns during embryonic and male germ cell development. PGC, primordial germ cell. Created with BioRender.com.

Germ cell-specific DNA methylation patterns are established at different time points for males and females. Male germ cells acquire male-specific DNA methylation marks for the most part premeiotically in the prenatal prospermatogonia in the period between midgestation and birth (Fig. 1). DNA methylation is nearly complete (~80%) by the pachytene phase of meiosis. Mitotic spermatogonia must also maintain the DNA methylation patterns acquired in the prenatal period. Further minor remodeling of DNA methylation occurs throughout meiosis and spermiogenesis. As most sperm-derived DNA methylation will be erased in the early embryo, it is postulated that germline DNA methylation, at sequences other than imprinted genes and repeats, may play specific roles in germ cell development, gene expression or chromatin structure during spermatogenesis. DNMT3A and DNMT3L are the major enzymes directing *de novo* DNA methylation in male germ cells; in mouse studies, mutations in the genes for these two DNMTs lead to male sterility. In rodents, DNMT3C targets ~1% of the genome (at young retrotransposons).

Transposable elements are parasitic sequences making up a significant part of the genome in higher eukaryotes (˜37% in mice and ˜45% in humans). The majority of these sequences have lost their ability to transpose except for the evolutionarily young ones. The young TEs need to be silenced by several pathways including DNA methylation in order to protect the genomic integrity of the organism. One of the important mechanisms leading to *de novo* DNA

methylation at these young TEs is the piwi-interacting RNA (piRNA) pathway. piRNAs are a class of non-coding RNAs highly expressed in male germ cells, and with their PIWI protein partners (e.g. MILI, MIWI2 and PLD6 in mice) they can recruit the *de novo* DNA methylation machinery to evolutionarily young retrotransposons in male germ cells. piRNA mutants and *Dnmt3C* mutant mice show a similar phenotype and demethylation of young retrotransposons along with the *Rasgrf1* imprinted locus indicating possible cooperation between them to keep these highly mobile TEs in check. It is not clear how humans are able to repress these young retrotransposons in the male germline since they lack DNMT3C.

DNA methylation plays a key role in the monoallelic expression of imprinted genes. Studies in mice and humans have shown that imprinted genes are not only crucial for prenatal development of the embryo and placenta but they are also required for the regulation of neurodevelopment, metabolism and physiological adaptations in the postnatal period. About 200 imprinted genes have been identified; changes in their expression and function can cause congenital diseases and, in some cases, increased cancer risk. Of the known imprinted genes, there are only three paternally (sperm) derived methylation imprints and over 20 loci that acquire methylation in the maternal germline (unmethylated in sperm). Imprinted genes provide an example of loci subject to epigenetic inheritance since their gamete-derived DNA methylation patterns can evade preimplantation demethylation and be passed on to the embryo and persist throughout development and/or life.

There is much current interest in the effects of infertility, the environment, and aging on human sperm DNA methylation and the potential for transmission of altered DNA methylation (epimutations) to the next generation. Until recently, most studies of DNA methylation in human sperm have targeted individual loci or a limited number of CpGs. Somatic cell contamination and genetic heterogeneity have emerged as key confounders in human sperm DNA methylation studies. Genome-wide DNA methylation analysis techniques such as whole genome bisulfite sequencing (WGBS), now allow DNA methylation to be assessed accurately at all CpG sites in the genome. WGBS profiles have been produced for the normal human sperm methylome and are starting to be used to assess alterations due to age and environmental exposures. Importantly, genome-wide DNA methylation analysis techniques such as WGBS probe methylation at genic, intergenic (including retrotransposons) and key regulatory sequences. High resolution sperm DNA methylation profiling with

genome-wide approaches and careful attention to confounders will be useful for prospective studies interested in connecting fathers' infertility/exposures to adverse outcomes in his children.

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Chapter 14
Do chromatin dynamics in spermatogenesis have implications for fertility and epigenetic inheritance?

Anne-Sophie Pépin and Sarah Kimmins

Male infertility is intertwined with environmental exposures including obesity, toxicants, and micronutrient deficiency. Remarkably the paternal environment also impacts the health of future offspring, potentially in a transgenerational manner. Evidence suggests that a connecting molecular link between environmental factors, male infertility and offspring health is the sperm epigenome. Alterations in metabolism associated with diet and obesity, or toxicant exposures can alter the sperm epigenome and in turn gene expression in spermatogenesis and the next generation leading to infertility and disease. However, we have a poor understanding at the molecular and genomic levels of how this occurs. The epigenome refers to the biochemical content associated with DNA and includes chromatin, organized as nucleosomes in which a section of DNA is wrapped around a core comprised of two of each histones H2A, H2B, H3 and H4. Each histone carries post-translational modifications such as phosphorylation, acetylation and methylation; these in turn influence the degree of chromatin compaction (e.g. euchromatin vs heterochromatin) and gene expression. While DNA can be thought of as storing genetic information, epigenetic processes interpret the genome, dictate spatio-temporal features of gene expression, are critical for cell differentiation, and are connected to human disease including male infertility. In general, the term epigenome includes three known layers of biochemical information: 1) the chromatin and specific patterns of post-translational modifications to histones; 2) DNA methylation which occurs at the 5-position of cytosine residues within CpG dinucleotides (Chapter 13); and 3) noncoding-RNA (Chapter 15). This chapter will focus on chromatin components of the sperm epigenome and its dynamic assembly during spermatogenesis and ability to impact fertility and disease intergenerationally.

Spermatogenesis is a highly complex cell differentiation process including proliferative mitosis and meiosis, and includes a massive remodeling of the chromatin in the haploid phase of spermiogenesis (Chapter 9). Unlike somatic cell development, the chromatin architecture of the male germline is highly varied as cells progress through differentiation. This highly specialized chromatin is specifically adapted to accommodate meiotic recombination, X chromosome inactivation, demarcation of imprinted genes, and the safe packaging of the genome and epigenome for delivery at fertilization. Disruption of the proper establishment and reprogramming of chromatin in spermatogenesis can have dire consequences for fertility and the health of subsequent generations (Fig. 1).

Unique to spermatogenesis is the incorporation of histone variants including those that are testis-specific (H1t, H2A.X., H2A.Z., H3.3, H2AL-1/2, HILS H3t, TH2A, TH2B). For example, beginning in the spermatogonia, the histone variant H3t gradually replaces the canonical histone H3. In meiosis, there is sequential incorporation of histone variants such as H1t, macroH2A.X, H2A.Z, and H3.3. In spermatocytes, the majority of histones H2A and H2B are replaced by the co-expressed germline-specific TH2A and TH2B. Interestingly, the combinations of H3.3 with macroH2A in spermatocytes and H3.3 with H2A.Z in round spermatids confer either more stable or unstable chromatin domains, respectively. The sex chromosomes are also enriched for variants and temporarily accumulate H2A.Z, which is subsequently replaced by H2A.B.3 at X-linked genes that escape sex chromosome inactivation. Localization of H2A.B.3 at the exon-intron boundaries of active genes, likely regulates RNA splicing events. Sex chromosomes also incorporate the replication-independent histone H3.3, which is encoded by genes *H3f3a* and *H3f3b*, both producing identical H3.3 but functioning at different stages of spermatogenesis.

A global reorganization in chromatin packaging occurs in spermiogenesis where most histone proteins are first replaced by the temporary transition proteins. Transition proteins (TP1 and TP2) are small basic proteins with overlapping functions and are important for chromatin condensation and DNA integrity. The incorporation of transition proteins onto chromatin is facilitated by the replacement of TH2A with variant H2A.L.2, promoting the opening of H2A.L.2-contaning nucleosomes. Their assembly on the genome ultimately permits the final eviction of histones and the incorporation of protamines. This histone-to-protamine exchange

begins in round spermatids by the weakening of interactions between histones and the DNA as a consequence of histone hyperacetylation. The high content in positively charged amino acids of protamines promotes compaction of the negatively charged paternal genomic DNA into supercoiled toroids. Compared to somatic cell heterochromatin, sperm DNA is ten-fold more compact due to this protamine facilitated organization.

Interestingly, 1% of sperm histones are retained in mice and about 10-15% in men. Retained histones are conserved across species from mice to men and are found at the regulatory regions of promoters implicated in spermatogenesis, sperm function, embryo development, metabolism and routine cellular processes. Histones are preferentially retained at high CpG enriched genomic regions that are predominantly DNA hypomethylated in the sperm of mice and men. Many of the retained canonical histones present in mature spermatozoa are enriched at key promoters and enhancers of developmental genes.

When interest in paternal epigenetic inheritance was emerging, it was unclear whether retained histones in sperm served specific functions and whether specific histones and their modifications were transmitted at fertilization. This changed when it came to be known that sperm histones were found to mark genes implicated in embryo development and suggested they may serve beyond gene regulation in spermatogenesis. This role of sperm transmitted histones has been demonstrated in mice. In a foundational study, it was shown that disrupting histone H3 lysine 4 dimethylation (H3K4me2) at transcriptional start sites in sperm had drastic consequences on the health of the next generations. Heterozygous transgenic male mice overexpressing the histone demethylase KDM1A in developing sperm were reproductively compromised and gave rise to offspring with severe developmental defects. These findings on the epigenetic landscape of mature spermatozoa and its evolutionary conservation, suggest sperm packages paternal information that is transmitted to the next generation and be instructive for developmental processes.

Environmentally-altered sperm chromatin has been linked to epigenetic inheritance through the paternal germline. Male mice fed a folate deficient or a high-fat diet have differentially enriched H3K4me3 in sperm at genes involved in developmental and metabolic processes that correspond to birth defects and metabolic dysfunction in their offspring, respectively. Regions with aberrant H3K4me3 in folate deficient sperm were retained in the pre-

implantation embryo and associated with alterations in embryonic gene expression. These studies demonstrated that diet-induced sperm H3K4me3 alterations are transmitted to the embryo and involved in environmentally-induced intergenerational phenotypes. Elucidating how other histone modifications are sensitive to environmental stress in sperm, escape epigenetic reprogramming in the embryo, and alter tissue gene expression and function will be critical to further understand the role of chromatin in paternal epigenetic inheritance.

In conclusion, there is strong evidence from mouse models that the unique chromatin composition in sperm is essential for spermatogenesis, can be responsive to the environment and can in turn alter embryonic gene expression, development and offspring health. Studies are needed to determine whether similar epigenetic mechanisms occur in men as this will open novel routes for the prevention and diagnosis of infertility, and the prevention of paternal disease transmission across generations.

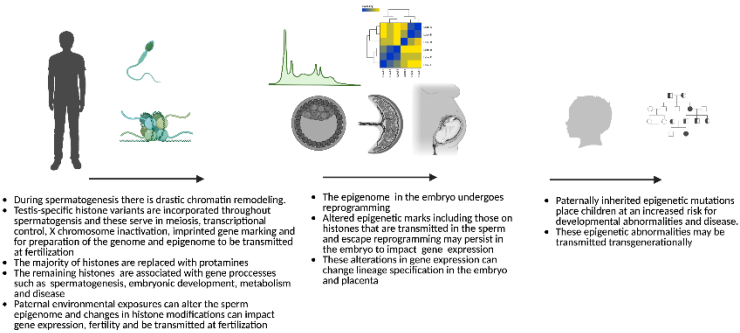

Figure 1. Spermatogenesis is characterized by unique chromatin remodeling that is implicated in fertility and intergenerational disease transmission.

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Chapter 15
What are sperm-borne RNAs and do they play a role in germ cell function?

Wei Yan

Due to a lack of typical cytoplasm, spermatozoa have been thought to contain no ribonucleic acids (RNAs). However, over the past fifteen years, numerous studies have demonstrated that sperm indeed contain RNAs, which can be delivered into the oocytes during fertilization. Given that RNAs can be obtained from either the whole sperm or sperm heads with or without the plasm membrane, the sperm-borne RNAs must exist in both the sperm nucleus and non-nuclear compartments. The sperm nuclear RNAs are likely loaded during late spermiogenesis when transcription is shut down and the spermatid nucleus starts condensation and elongation, whereas the RNAs located in the non-nuclear compartments of the sperm, e.g., perinuclear theca, neck and flagellum, may derive from either the former cytoplasm, which gets shed upon spermiation (i.e., sperm release from the seminiferous epithelium), or from the cytoplasmic droplets, representing a highly specialized organelle as an integral part of the maturing epididymal sperm. Recent data suggest that sperm gain small RNAs from the exosomes of the epididymal epithelial cells when sperm transit through the epididymis for maturation, but the claim was primarily based on the dynamic changes in proportional distribution patterns of small RNAs in testicular, caput, and cauda epididymal sperm. Given that epididymal sperm mostly contain cytoplasmic droplets, which also contain a large number of large and small RNAs, it is imperative to examine whether the changing small RNA compositions between sperm from the caput and cauda epididymides are due to small RNA shuffling between the cytoplasmic droplets and sperm, or between the epididymosomes and sperm. Nevertheless, the sperm RNA payloads appear to be selective because the species and relative abundance of various RNAs differ between the sperm nucleus and non-nuclear compartments. It remains unknown how specific RNAs are selected and loaded into the sperm nucleus and other compartments during spermiogenesis and epididymal maturation.

Sperm contain both large (>200nt) and small (<200nt) RNAs, but sperm RNAs are much less abundant compared to those in somatic cells (~0.1-0.3 pg per human sperm vs. 10-30 pg in a typical somatic cell) (Fig. 1). Among the sperm-borne large RNAs, messenger RNAs (mRNAs), large noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) have been detected. Although full-length mRNAs have been identified through the latest third-generation long-range sequencing, most mRNAs in sperm appear to be truncated mRNA fragments in both linear and circular forms. Many sperm-borne circRNAs contain open reading frames, implying their potential to be translated into proteins. Indeed, peptides corresponding to these sperm-borne circRNAs have been identified through proteomics analyses. However, it remains to be determined whether these peptides are the products of the circRNAs. Given that circRNAs are resistant to degradation, it has been proposed that these circRNAs may function to maintain continued protein production when mRNAs are undergoing massive decay toward the end of spermiogenesis and are largely unavailable after spermiation.

Sperm-borne small RNAs are relatively more abundant than large RNAs in sperm. Numerous small RNA species have been identified, including microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), piwi-interacting RNAs (piRNAs), tRNA-derived small RNAs (tsRNAs), rRNA-derived small RNAs (rsRNAs), mitochondrial genome-encoded small RNAs (mitosRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and many other unannotated small RNA species (Fig. 1). The relative abundance of various small RNAs species differs between whole sperm and sperm heads. For example, tsRNAs are more enriched in the sperm head, whereas mitosRNAs are always more abundant in the whole sperm. The differential small RNA distribution patterns within a sperm further support the notion that the sperm-borne small RNAs are selectively loaded into the nucleus and other non-nuclear compartments. Of interest, the sperm-borne small RNA profiles are different from those in their precursor cell types (e.g., spermatids), but appear to be conserved among closely related mammalian species, further supporting the concept that these small RNAs are purposely packaged into sperm and may have conserved physiological roles.

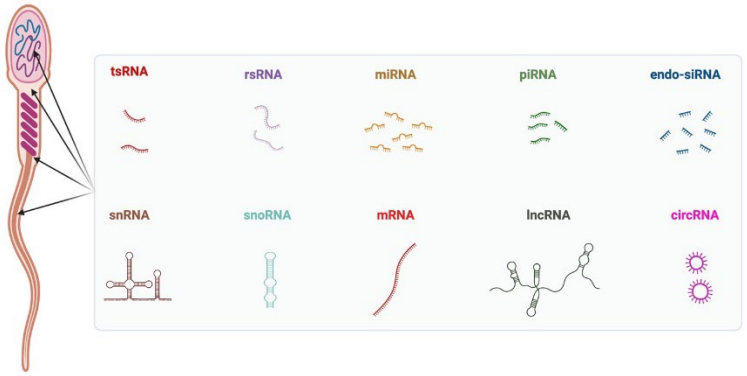

Figure 1. Types of RNAs and their locations in mammalian sperm.

Although sperm contain full-length mRNAs, it is highly unlikely that these sperm-borne mRNAs can be translated into proteins due to a lack of cytoplasm and the canonical, cytoplasmic translational machinery. A previous study reported that sperm may be able to translate their mRNAs into proteins using the mitochondrial translation machinery, but subsequent independent studies have failed to validate this claim. Alternatively, the sperm-borne mRNAs, in theory, can be translated into proteins upon delivery into the oocyte cytoplasm, thereby participating in fertilization and preimplantation embryonic development. However, this has not been demonstrated experimentally. Similarly, the potential functions of sperm-borne large noncoding RNAs remain to be investigated.

Sperm-borne miRNAs and endo-siRNAs have been shown to be important for fertilization and preimplantation embryonic development because partial depletion of miRNAs and endo-siRNAs in sperm causes compromised early embryonic development due to dysregulated transcriptomic profiles in fertilized eggs and early embryos. Since miRNAs and endo-siRNAs are known to function to regulate mRNA stability through complementary binding to the 3'UTRs of mRNAs, the dysregulated transcriptome of early embryos may result from sperm deficiency in miRNAs and endo-siRNAs. In addition to the canonical role of miRNAs and endo-siRNAs, sperm-borne small RNAs appear to mediate non-genetic inheritance of acquired paternal traits, including the disease phenotypes induced by environmental and dietary factors (e.g., glucose intolerance and

metabolic disorders induced by a high fat or a low protein diet,) and certain paternal behavior gained during the father's lifetime. More intriguingly, microinjection of mouse sperm-borne total RNAs, miRNAs, tsRNAs or rsRNAs into wild-type mouse oocytes appears to be able to recapitulate the paternal phenotypes in offspring, suggesting these sperm-borne small RNAs may function as the epigenetic information carrier responsible for the paternal transmission of the phenotypes acquired during a father's lifetime. However, it remains unclear how sperm-borne small RNAs act at molecular levels to induce the specific paternal phenotypes in offspring.

In summary, mammalian sperm contain RNAs, both large or small and coding or noncoding (Fig. 1). These RNAs are likely purposely loaded into sperm during spermatogenesis. Sperm-borne RNAs may have roles in supporting preimplantation embryonic development and as one of the epigenetic information carriers to mediate epigenetic inheritance of paternally acquired traits through environmental, dietary, and other factors.

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Chapter 16
How are epigenetic marks transmitted from one generation to the next?

Epigenetics, intergenerational inheritance, transgenerational inheritance, chromatin, DNA methylation, histone modifications, noncoding RNAs, germ cells, gametes, embryos

John R. McCarrey

Inheritance can occur in either of two forms – genetic or non-genetic. The latter is termed epigenetic inheritance. Genetic inheritance refers to semi-conservative replication of DNA which faithfully copies the template DNA sequence present in the double helix structure of the chromosomes of a parent cell (the genome) into two double helix copies to be transmitted to daughter cells during mitosis or meiosis I. Epigenetic inheritance refers to transmission from parent cells to daughter cells or generation to generation of reversible modifications to the DNA or associated proteins or of noncoding RNAs associated with the DNA (the epigenome), and is based on different mechanisms depending on which epigenetic modification is being copied or transmitted. All of these mechanisms function to transmit information from one generation to the next, and this information is critical for proper development and function of each subsequent generation, but is also subject to disruption by environmental or lifestyle effects.

Epigenetic marks

Epigenetic marks (also known as – epigenetic programming) are manifest in a variety of forms. In each case, the pattern of these marks is normally heritable through mitosis or meiosis and it contributes to regulation of gene expression by influencing chromatin structure. Methylation of the 5-carbon position of cytosines (Cs) present in 5'-3' CpG dinucleotiedes in mammalian DNA to form 5-methyl cytosine is perhaps the best studied epigenetic mark (Chapter 13). An absence or low level of DNA methylation (hypomethylation) is typically associated with a decondensed chromatin

state and active or primed gene transcription, while presence or a high level of DNA methylation (hypermethylation) is typically associated with a condensed chromatin state and repressed gene transcription. A well-understood mechanism termed "maintenance DNA methylation" normally faithfully propagates the presence of DNA methylation patterns during DNA replication (Fig. 1). A 5'-3' CpG dinucleotide in one DNA strand will normally be complemented by a 3'-5' CpG dinucleotide in the opposite strand of a double helix. A "fully methylated" site will have methylated Cs in the CpG dinucleotides in both strands. Upon semi-conservative DNA replication, an unmethylated C will be inserted in the new CpG dinucleotide complementary to the existing methylated or unmethylated CpG dinucleotide in the parent or template DNA strand. If the C in the template strand was methylated, this will create a transient "hemimethylated" state in which the C in the parent strand is methylated but that in the newly synthesized strand is unmethylated. However, the DNMT1 maintenance DNA methyltransferase which is normally associated with the DNA replication complex recognizes this hemimethylated state and inserts a methyl group onto the unmethylated C, restoring the site to fully methylated status. If the original site was unmethylated (no methylated C on either strand) then it will remain unmethylated following DNA replication since no transient hemimethylated state will be achieved at any point. In addition to maintenance DNA methylation, other DNA methyltransferases can catalyze "de novo" methylation which results in a previously unmethylated CpG site (no methylation on the C in the CpG dinucleotides on either strand) becoming fully methylated (= the Cs in the CpG dinucleotides on both strands becoming methylated). Finally, it is also possible for a fully methylated site (methylated Cs on both strands) to become "actively demethylated" such that neither C remains methylated. Active demethylation is normally catalyzed by the TET enzymes.

Other epigenetic marks include a variety of different post-translational modifications of histones in nucleosomes. These include both methylation or acetylation, as well as several other modifications including phosphorylation, ribosylation, sumolation, and ubiquitination, among others. Like DNA methylation, histone modifications are known to influence chromatin structure – either promoting chromatin condensation which represses transcriptional activity or chromatin decondensation which activates or primes transcriptional activity. They do this either directly by influencing

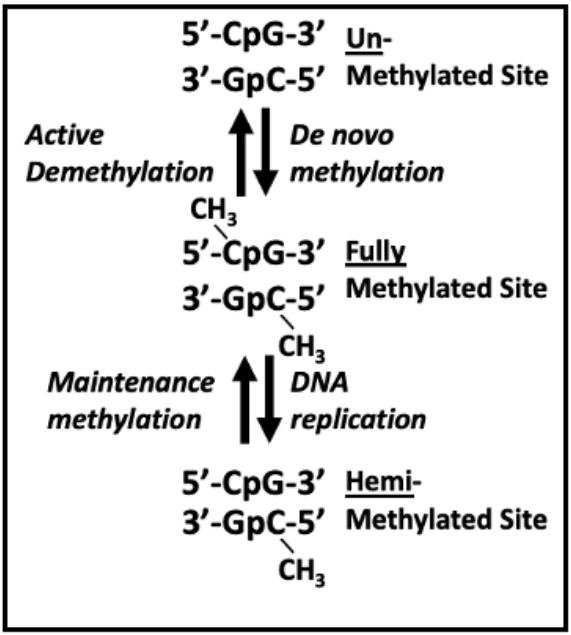

Figure 1. Epigenetic inheritance of DNA methylation states. In mammalian genomes, DNA methylation normally occurs at 5'-CpG-3' dinucleotides. A CpG dinucleotide in one strand of double-stranded DNA will be paired with a CpG dinucleotide in the opposite strand. If neither CpG is methylated the site is "unmethylated." If both CpGs are methylated, the site is "fully methylated." During semi-conservative DNA replication of a fully methylated site, the newly synthesized strand will initially carry an unmethylated CpG creating a transient "hemimethylated site." The hemimethylated site is recognized by the DNMT1 maintenance DNA methyltransferase which adds a methyl group to return the site to fully methylated status, thereby facilitating epigenetic inheritance of a fully methylated site. An unmethylated site can become directly methylated on both CpGs by a process called "de novo methylation." A fully methylated site can become directly unmethylated by a process called "active demethylation."

charge and, hence, affinity among neighboring nucleosomes, or indirectly by attracting chromatin remodeling complexes in a non-sequence-specific but a histone modification-specific manner (Chapter 14). Finally, non-coding RNAs, including both long and

small ncRNAs, also contribute to regulation of chromatin structure, although the mechanism(s) by which this is accomplished are less well understood. ncRNAs have the potential to serve as "sequence guides" when their sequence is complementary to that in the target DNA (Chapter 15). Empirical evidence indicates that patterns of histone modifications and/or ncRNAs are heritable. However, the molecular mechanisms responsible for faithful propagation of these patterns are not well understood. Importantly, the epigenetic programming that regulates cell-type specific gene expression patterns is manifest on the basis of the cumulative patterns of all of these epigenetic marks. Ultimately, cell-type specific gene expression patterns are regulated by transcription factor networks operating within 2-dimensional chromatin landscapes that function within a 3-dimensional interactome within the cell.

Epigenetic programming/reprogramming

Epigenetic marks are, by definition, reversible. Their patterns differ in each cell type, and further vary as a function of development, aging, influences from the environment, and/or disease states. Importantly, epigenetic programming undergoes major periods of erasure and resetting during each generation. This is termed "epigenetic reprogramming." Epigenetic programming contributes to regulation of cell-type specific gene expression which, in turn, directs cell-type specific differentiation. The epigenome serves as a liaison between the environment and the genome. This includes sensing position within the developing embryo and fetus to direct proper development of different cell types within the body, as well as mediating subsequent changes during development, aging, environmental or lifestyle effects, or disease states. Intergenerational transmission of epigenetic programming is mediated primarily by the germ line and early embryo. Germline development and gametogenesis are regulated by mechanisms similar to those that control tissue- or cell-type specific gene expression in any somatic cell type, resulting in production of spermatozoa in males and ova in females. However, unlike somatic cell differentiation, germline differentiation is not a terminal process given the function of the gametes which give rise to the next generation. Thus, germline cells must undergo extensive cell-type specific differentiation to form the gametes, but the resulting gametes must retain the potential to form, upon unification via fertilization, an entire new embryo and individual. This mandates the need for major phases of epigenetic

reprogramming in the early embryo and germ line (Fig. 2). Thus, in the early embryo, epigenetic programming that was associated with production of the gametes is largely, and rapidly, erased following fertilization. Subsequently, a new wave of epigenetic programming is established by the time of implantation which predisposes specification of the germ layers and the germ line. Certain inherited,

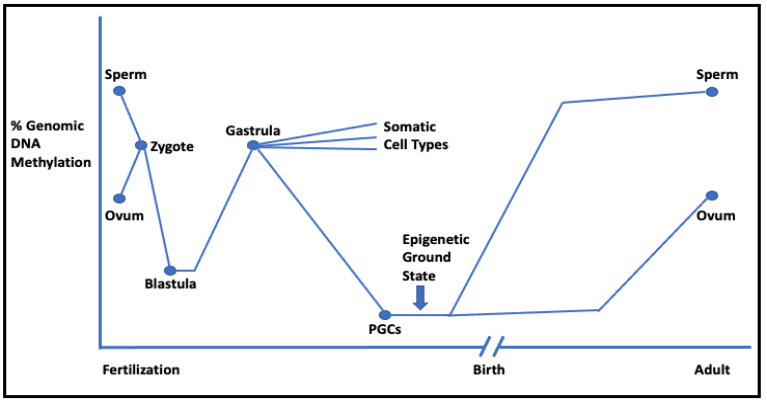

Figure 2. Dynamic, genome-wide changes in DNA methylation throughout the mammalian lifespan exemplified in the mouse. Genome-wide levels of DNA methylation undergo dynamic transitions throughout the lifespan of an individual. These include embryonic reprogramming in the preimplantation embryo in which DNA methylation inherited from the sperm and ovum is largely erased by the time the blastula is formed. This is followed by re-establishment of genome-wide DNA methylation at about the time of gastrulation in precursors of both the soma and germ line. Locus-specific epigenetic programming in precursors of specific somatic cell lineages then contributes to development of each somatic cell type, with no further genome-wide reprogramming of DNA methylation. A second major wave of genome-wide demethylation occurs during the unique process of germline reprogramming which is even more extensive than that which occurs during embryonic reprogramming, leading to the epigenetic ground state. This is followed by resetting of DNA methylation patterns in the developing germ cells to produce gametes carrying epigenetic programming needed for development of the subsequent generation. Figure modeled after Lee et al. (2014).

parent-of-origin, allele-specific differences in epigenetic programming (at imprinted loci) are retained throughout this embryonic reprogramming process. However, uniquely in the developing germ line, there is a second wave of even more extensive epigenetic reprogramming that includes erasure of unique programming inherited from each parent resulting in the "epigenetic ground state" . This is followed by yet another wave of de novo epigenetic programming that is similar on both copies of the genome in each developing germ cell such that each haploid gamete in any individual ultimately carries similar programming that will be transmitted to the next generation.

Transgenerational epigenetic inheritance of epimutations

Whereas the genome is a highly stable entity which is protected by DNA repair mechanisms that significantly mitigate the occurrence of genetic mutations, the epigenome is a highly labile entity that is, by definition, reversible and responsive to changes in the environment or the lifestyle or health of the individual and lacks the benefit of any sort of repair mechanism other than reprogramming. As a result, there is a significant potential for disruption of normal epigenetic programming due to aberrant environmental, lifestyle or health conditions, resulting in abnormalities known as "epimutations." Abundant evidence now exists in support of the potential for exposure to disruptive substances such as endocrine disrupting chemicals (EDCs), or aberrant lifestyle choices or imposed circumstances such as famine or dietary deficiencies to induce epimutations in both the soma and germ line. Because epigenetic progamming is heritable, epimutations induced in one generation can potentially be transmitted to subsequent generations. This has now been clearly demonstrated by multiple studies. Theoretically, exposure to a disruptive environmental influecnce could induce an epimutation via a direct toxic effect that might not be heritable. However, studies have now demonstrated that a single exposure of an F0 generation pregnant female and the F1 fetuses she is carrying can result in defective phenotypes in not only the directly exposed F1 offspring, but also in subsequent generations, even in the absence of any subsequent exposure to the disruptive influences. Actually, development of epimutant phenotypes in F2 generation offspring of an exposed pregnant female could also represent a direct, non-heritable, toxic disruption of the epigenome in the primordial germ

cells (PGCs) present in the F1 fetuses carried by the F0 pregnant female, since those PGCs will give rise to the F2 generation. However, studies have shown that a single exposure of an F0 pregnant female can induce and predispose multigenerational transmission of epimutations beyond the F2 generation – to the F3, F4 or even F5 generations – with no further exposure to the initial causative effect. Additional studies have shown that these exposures are not inducing genetic mutations. Thus, this represents initial disruption of normal epigenetic programming to generate epimutations that are subsequently transmitted to multiple descendant generations via epigenetic inheritance. This is termed transgenerational epigenetic inheritance of environmentally-induced epimutations.

Initial disruptions of the epigenome that are subsequently transmitted to daughter cells via mitosis or generations via meiosis via epigenetic inheritance are termed "primary epimutations." An alternative mechanism by which similar, heritable phenotypic effects can be induced involves an initial genetic mutation that disrupts a normal mechanism by which epigenetic programming is established, maintained and/or propagated. An example would be a defect in a DNA methyltransferase or a histone modifying enzyme which, when defective, fails to establish proper epigenetic programming. Epimutations of this sort are termed "secondary epimutations" and can be transmitted by either genetic or epigenetic inheritance, or both. A third type of epimutations are those that arise as a secondary effect following induction of primary epimutations that, in turn, induce defects in mechanisms that normally maintain genetic integrity (e.g. DNA repair and/or cell death mechanisms), and therefore predispose a subsequent abnormal increase in the accumulation of genetic mutations. These are termed "tertiary epimutations."

Summary

Epigenetic programming is critical to normal cell, tissue and organ function because it regulates unique gene expression patterns required for each differentiated cell type. Epigenetic programming is also heritable and so is normally transmitted during DNA replication, cell division and intergenerational or transgenerational reproduction. It is reversible, and, unlike the genome, the epigenome is different in every cell type and undergoes dynamic changes during development and aging. A unique feature of normal function of the

epigenome is its ability to undergo modifications in response to cues from the environment. This is critical to the normal function of epigenetic programming, but also renders the epigenome susceptible to abnormal disruptions which, given the heritability of epigenetic programming, can be transmitted from generation to generation. Interestingly, past commonly accepted attitudes toward proper parental health held that while a future mother should be cognizant of her lifestyle choices, even prior to conception of her offspring, a future father did not need to worry about any pre-conception exposures or lifestyle choices as long as they did not threaten the genetic integrity of his gametes. We now know that this perception was naive and that a father transmits both his genetic and epigenetic information to his offspring and must therefore be cognizant of his potential pre-conception exposures or lifestyle choices to the same extent as a future mother.

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Chapter 17
What are the efferent ductules?

Rex A. Hess

Are efferent ductules part of the testis?

Efferent ductules are the numerous small, thin-walled, highly convoluted tubules that connect rete testis with the single tubule called the epididymis. Historically the efferent ducts have received far less attention than the epididymis in the male reproductive tract. However, sperm produced in copious amounts must exit the testis in a dilute physiological fluid and be transported into the epididymis for maturation, storage and then ejaculation. It is essential that transport from the testis be rapid and without obstruction, otherwise fluid will back up into the rete testis and seminiferous tubules, producing a cascade of male reproductive problems, including infertility. This important passage is dependent on the rete testis that forms chambers within the mediastinum of the testis, in man, and the series of small tubules called the efferent ductules (or ducts). The rete testis network of channels or chambers join with individual efferent ductules at the testicular surface or the tunica albuginea. The rete epithelium changes dramatically from a flattened, variable height structure to a low columnar epithelium in efferent ducts. The efferent ducts are not part of the testis, anatomically, as they reside outside the fibrous tissue capsule that covers the testis and are derived embryologically from the mesonephros. However, the answer regarding efferent ducts and the epididymis is less straightforward.

Are efferent ductules part of the epididymis?

Efferent ducts are derived embryologically separate from the rete testis, first forming as mesonephric tubules that grow out from the Wolffian duct towards the rete testis cords. Thus, it might be assumed that efferent ducts are also separate and distinct from the epididymis, which is specifically a Wolffian duct-derived tube. The problem of classification arises for two reasons: a) In man, numerous mesonephric tubules that eventually become the efferent

ducts occupy the entire head of the epididymis, the region closest to the testicular hilum, with most of the efferent ducts opening individually into the proximal region of the epididymis (Fig. 1); b) In rodents, the cranial mesonephric tubules grow out from the Wolffian duct and connect individually with the rete testis cells, but new research has revealed that before birth the folded, cranial portion of the Wolffian duct becomes the "common efferent duct" into which all the efferent ductules open. In adult rodents, the common efferent duct is enclosed by the caput epididymal connective tissue capsule and empties into the initial segment of the epididymis (Fig. 1). Thus, it can be argued that at least a portion of the efferent ductules, the common duct, is embryologically related to the epididymis, at least in rodents. In man, the entire proximal (caput) epididymis (gross anatomically speaking) is occupied by the efferent ductules. Therefore, yes, the efferent ducts should be classified as a subsection of the caput epididymis. In man, this would include the entire proximal caput epididymis and in rodents this portion could be further subdivided into proximal efferent ducts, a conus region and the common duct. Early literature has at times made this association, with statements such as, "Efferent ducts of the human epididymis..." Even in the rat, early work included efferent ducts as part of the overall classification of the term "epididymis".

Structure and function of efferent ductules

Although it is reasonable to label efferent ducts as a subsection of the caput epididymis, their epithelial morphology and general physiology set them apart distinctly from the main body of the epididymis. This feature alone does not prevent the ductules from being classified as part of the epididymis, because epithelial morphology even of the epididymis in some species varies dramatically from caput to cauda. There is an abrupt epithelial change from rete testis to efferent ductules and from efferent ducts to the epididymis tubule. Rete testis epithelium is squamous to low columnar in height, with few microvilli and a single primary cilium (non-motile), while the efferent duct epithelium is abruptly taller, columnar in shape and lined by two cell types, ciliated (motile cilia) and non-ciliated with numerous short microvilli and also a primary cilium (Fig. 2). The epididymal epithelium is also recognized by its abrupt change into a tall, pseudostratified columnar epithelium, with long, branched microvilli, but no motile cilia. However, there is considerable variation in cell morphology along the length of the ductules, with

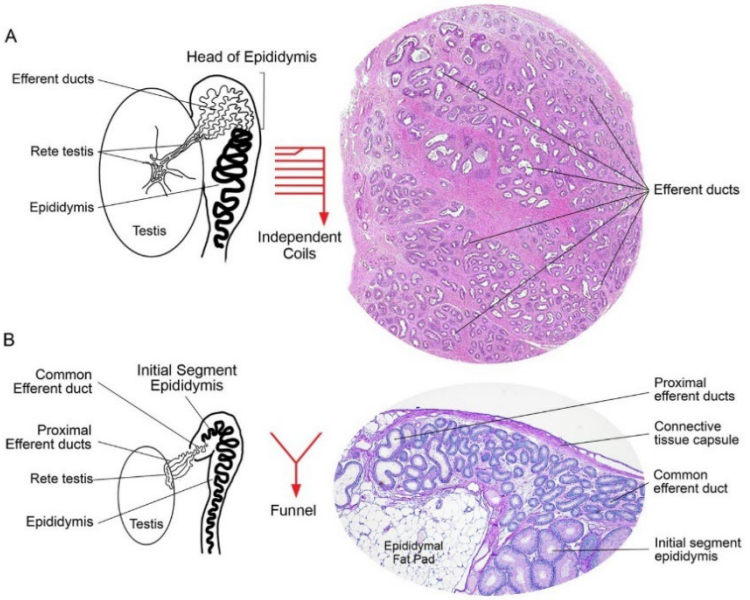

Figure 1. Two basic types of efferent ductule organization are illustrated. **(A)** "Independent coils" are found in man and larger mammals. The individual efferent ducts extend from the rete testis and most stay separated, with only a few ducts merging before opening into the epididymis. The numerous efferent ducts (approximately 15) occupy the entire proximal or head of the epididymis. Thus, there are numerous separate entries of the efferent duct lumens into the caput epididymis. Histology of the epididymal head region in man is shown on the right, with cross sections of the coiled separate ductules surrounded by thick areas of connective tissue (photo provided by Dr. Robert Sullivan, CHU de Quebec-Université Laval Research Center, Québec, QC, Canada). There is considerable variation in the luminal diameters. **(B)** The "Funnel" formation is present in rodents and smaller mammals. Individual efferent ductules (2-8) carry sperm from the rete testis, but all merge to eventually form a single, common duct that enters the head of the epididymis and opens into the initial segment. Thus, the seminiferous tubular fluid that is not reabsorbed by the efferent duct epithelium is funneled into a small, single tubule. Histology of the mouse efferent ductules is shown on the right. The proximal ducts, nearest the rete testis, have a wider lumen than those in the conus (area of merging) and common duct regions. The common duct resides under the connective tissue capsule that encloses the epididymis and opens into the larger initial segment.

species variations. The major function of efferent ducts is the reabsorption of nearly 90% of the luminal fluid, which increases the concentration of sperm as they enter the initial region of the epididymis. This function is reflected in the two epithelial cell types. Nonciliated cells have an absorptive microvillus border with endocytotic vesicles and lysosomal granules, while the ciliated cells support long motile cilia that agitate or stir the luminal fluids, which ensures the lumen does not become clogged as the sperm become more concentrated.

In man, sperm are transported through the epididymis in 1-5 days, but transit in the efferent ducts occurs within hours and even less than 1 hour in the rat. Therefore, the structure and function of these ductules must be highly regulated and well organized to avoid sperm stasis and occlusions. The ducts are organized into two basic designs (Fig. 1). In man and other larger mammals, the ductules

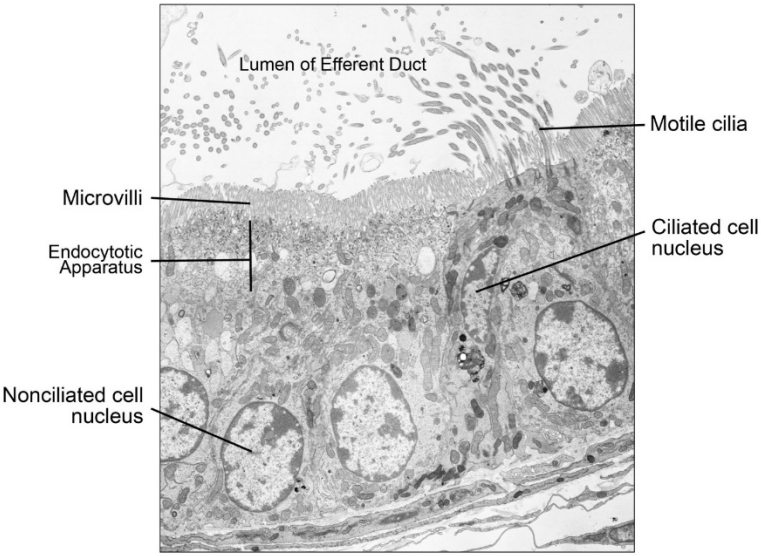

Figure 2. Efferent ductule epithelium consists of two cell types. Shown is a transmission electron microscopy section from the mouse, which is typical for all species. The nonciliated cell is lined by a short microvillus border at the lumen, which is highly absorptive as demonstrated by a prominent endocytotic apparatus. The ciliated cell supports long motile cilia that extend from basal bodies into the lumen for the purpose of stirring or agitating the luminal fluids.

form "*independent*" convolutions that occupy most of the proximal or caput epididymidis. This arrangement consists of approximately 15 adjacent strands of coiled efferent ducts extending from the rete testis, with most of these ductules remaining independent, rather than merging, and thus providing multiple entries into the initial region of the epididymis. The other basic formation is that of a "*funnel*", which is found in smaller mammalian species, including rodents. Efferent ducts with the funnel organization open separately from the rete testis (2-8 proximal ducts) but all of the ducts then begin to merge until they form a single, very thin diameter, common ductule that penetrates the epididymal connective tissue capsule for emptying into the initial segment epididymis. Thus, rapid reabsorption of luminal fluid is required to prevent overloading the volume capacity of the common duct, because all of the un-reabsorbed seminiferous tubular fluid is funneled along with the sperm into this small, single ductule. This difference in efferent ductule organization is rather significant and highly relevant to reproductive pathology in both man and other species.

Regulation and potential pathology

Androgens and the androgen receptor (AR) pathway are well known for their regulation of development and adult function of the epididymis. However, surprisingly the efferent duct epithelium is dependent on the estrogen receptor-1 (ESR1) pathway, as well as AR, for both development and function, particularly the ability to reabsorb nearly 90% of the luminal fluid. How might this discovery impact our interpretation of human epididymal pathology? Lesions are more commonly found in the caput region of the epididymis, along with the formation of sperm granulomas, which experimentally are related to disruptions in fluid reabsorption, as well as loss of ESR1 activity. A potential problem arises because most research is performed in rodent species, which carry the "funnel" organization, and thus the efferent ducts are more susceptible to total blockage. Complete occlusion of the common duct will result in azoospermia and back-pressure atrophy of the testis, a commonly reported pathology in rodent models. In man, the ductules do not funnel into a single duct and therefore, although sperm stasis and granulomas can form, there is less likelihood of total blockage and testicular atrophy. This difference between smaller and larger species raises numerous questions and requires careful extrapolation of rodent studies to man. Finally, further

investigation is warranted into the potential effects on epididymal sperm and fertilizing ability in cases of partial disruption or blockage of some but not all efferent ductules.

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Chapter 18
What does the epididymis do and how does it do it?

Clémence Belleannée and Barry 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 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 by providing an appropriate luminal fluid microenvironment. The paternal genome being transported is in an ultracondensed and inactive form in epididymal spermatozoa; the acquisition of sperm fertilizing ability relies exclusively on molecules deriving from the testis (lumicrine factors) as well as from factors secreted by the surrouding epididymal epithelial cells. The challenge for many investigators has been to identify those molecules. In addition to its sperm maturational role, the epididymis creates an immune priviledge environment that protects spermatozoa against infection and immune attack as they mature (Chapter 20). It also provides an environment for maintaining mature spermatozoa stored in a quiescent state until ejaculation. Since spermatozoa are immotile, they require assistance to move along this very long duct. This movement is aided by contractions of smooth muscle cells that surround the duct as well

as pressure from fluid and spermatozoa entering the duct from the testis. From a clinical perspective, an malfunctioning 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.

Development of the epididymis

The intermediate mesoderm gives rise to the mesonephros, that in turn, gives rise to the mesonephric duct (Wolffian duct), Müllerian duct and mesonephric tubules. Under the influence of testosterone and anti-Müllerian hormone, the Müllerian duct degenerates leaving the Wolffian duct, which throughout development undergoes extensive elongation and coiling. The mechanisms by which the Wolffian duct elongates includes cell proliferation, cell intercalations, and biomechanical forces derived from the surrounding capsule and the extracellular matrix. Protein tyrosine kinase 7, inhibin βA, β-catenin, PLAG1, members of the sonic hedgehog and Hippo pathways, and SPAG11c, a β-defensin, are genes that have been shown to regulate elongation and coiling.

The gross anatomical structure of the epididymis in a variety of species is divided into several regions that include: the initial segment (observed in rodents), caput, 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.

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). 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, proteins and transport water through aquaporin channels as a mechanism to concentrate spermatozoa.

Structure of the epididymis

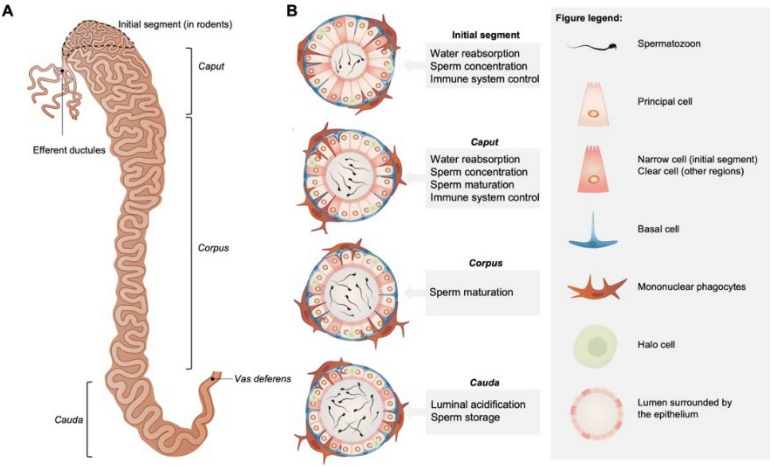

Figure 1. Structure, cellular organization and functions of the epididymis. (A) Schematic representation of the different regions of the epididymis: initial segment, caput, corpus and cauda. **(B)** Cross-sectional representations of the epididymal duct at each region. The major cell types and related functions are presented based on observations chiefly done in rodents. 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

They also participate in the release of extracellular vesicles following the formation of apical bleds from the apical membrane. These vesicles transfer bioactive molecules, including small non-coding RNAs and proteins, that participate in the control of sperm fertilizing ability and in the transmission of paternal traits acquired by environmental conditions to the offspring. 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 the maintenance of mature spermatozoa in a quiescent state. Basal cells display stem-cell like properties and form primary cilia as well as long axiopodia that sense and monitor the composition of the extracellular envrionment. The function of apical cells is unclear; however, there is evidence demonstrating that they endocytose material from the epididymal lumen. Halo cells are a mix of T lymphocytes, monocytes

and cytotoxic T-lymphocytes and may have a role in immune protection. Furthermore, a dense network of mononuclear phagocytes surrounds the epididymal epithelium and extends slender projections towards the lumen to control the immune environment. Surrounding the entire duct are layers of smooth muscle/myoid cells 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. Single cell sequencing data performed on human and mouse tissues further extended the molecular and cellular complexity of the epididymis and will help improve our knowledge on the mecanisms controlling sperm maturation.

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 basalateral 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 the initial segment to the 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, Dicer1 conditional knock-out, and Foxi1 null. Foxi1, a transcription factor, is of particular interest because it is known to regulate the expression of vacuolar H+-ATPase protion pump, carbonic hydrase 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.

Conclusion

In summary, the epididymis promotes sperm maturation, facilitates the transport of spermatozoa along the duct, stores spermatozoa and protect them from harmful substances. All of these functions are coordinated with remarkable precision to ensure production of fully viable, functional spermatozoa.

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Chapter 19
What is the impact of the immune system on male reproductive function?

Mark P. Hedger and Rukmali Wijayarathna

The immune system in the context of male reproductive biology

The primary role of the immune system is to protect its host from disease and facilitate repair following injury or organ damage. It does this via a complex network of leukocytes, secretions and receptors that support active immunity. Immunity is based on the recognition of molecules specifically expressed by pathogens or produced only during tissue damage (innate immunity), or through encountering structural motifs (antigens) that are not normally expressed during normal physiological functions, and are therefore considered to be "foreign" and potentially dangerous (adaptive immunity). This ability to discriminate foreign from "self" antigens, called immunological tolerance, is principally established during fetal and perinatal life. Consequently, spermatogenesis presents a unique problem for immunity, in that many molecules involved in spermatogenesis are not present during early development, and only arise during sexual maturation. As a result, the male tract and sperm especially are susceptible to damage by the immune system.

Crucially, the male reproductive organs are open to the external environment via the urogenital tract. Consequently, ascending infections, usually bacterial in nature, travelling through the urethra and up to the accessory glands, epididymis and testis, are common. These can be sexually transmitted (*e.g. Chlamydia trachomatis*, *Neisseria gonorrhoeae*), but the majority of such infections are due to commensal organisms (*e.g. Escherichia coli*, *Ureaplasma urealyticum*). The testis is also particularly susceptible to a number of sexually transmitted and even systemic viral infections, including mumps, human immunodeficiency virus (HIV), hepatitis B, and many emerging viruses, such as *Zika*. These infections commonly cause pain, cell and tissue damage and may lead to infertility.

The principal cell types responsible for immunity are the mononuclear phagocytes (macrophages and dendritic cells), lymphocytes (T cells, B cells and natural killer cells) and polymorphonuclear cells, such as neutrophils (Fig. 1). Fundamentally, the immune system is triggered when the mononuclear phagocytes respond to a perceived threat, either a known pathogen (via molecular pattern recognition receptors) or upon encountering a foreign antigen (via antigen-specific T cell and B cell receptors). The mononuclear phagocytes and neutrophils become activated very rapidly, secreting cytokines, complement factors and other molecules that induce inflammation (innate immunity). Inflammation is characterised by increased blood flow to the site of infection or injury, the recruitment of immune cells from the blood into the tissue and the production of cytotoxic substances and pain. This leads to tissue damage, which needs to be repaired during the resolution phase, once the immunological stimulus has been cleared. During inflammation, antigen-specific T cells are recruited and interact with the macrophages and dendritic cells, in a process called antigen-presentation, principally in the lymph nodes or other secondary immune tissue, such as the spleen. The activated T cells then target infected cells for killing, or activate antibody-producing B cells. This second phase involving T and B cells (adaptive immunity) usually takes several days to become effective, unless the host has been exposed to the antigens before through prior infection or immunisation.

Central tolerance occurs mainly in the thymus during early development, via clonal deletion to eliminate self-reactive T cells and the differentiation of regulatory T cells (Treg cells). These processes may not succeed in controlling autoreactivity in all circumstances and self-reactive T cells may persist or become activated later in life, resulting in autoimmune disease. Therefore, peripheral tolerance processes are important, wherein self-reactive T cells become functionally unresponsive (anergy) or are deleted when self-antigens are encountered outside of the thymus, throughout life. This involves continuous low-dose exposure of the self-reactive cells to their antigen.

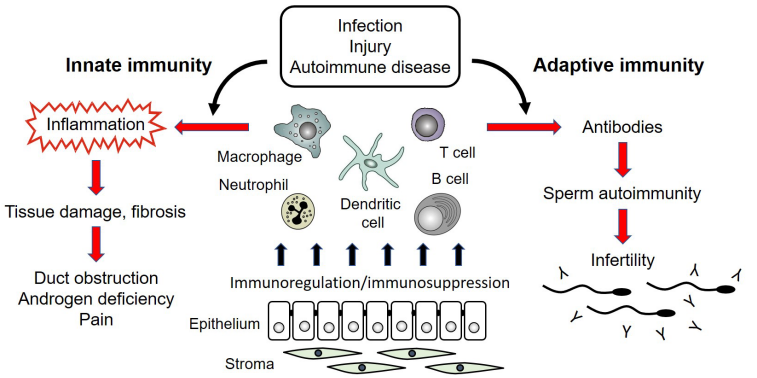

Figure 1: Diagram summarising the two arms of the immune system (innate and adaptive immune responses) activated by infection, injury or autoimmune disease in the male reproductive tract. Resident and circulating immune cells are normally under the regulatory influence of the epithelial and stromal cells, but immune activation leads to dysregulation of this control. This results in inflammation and damage to the cells and tissue of the male tract and the formation of sperm antibodies, causing temporary or chronic pain and infertility.

Immunoregulation and immune privilege in the testis

Spermatogenesis occurs within the seminiferous epithelium, which is avascular. All testicular blood vessels and lymphatics are located outside the seminiferous tubules in the interstitial tissue, and this is where the immune cells are also found. These include resident macrophage populations, which are found surrounding the tubules (peritubular macrophages) and throughout the interstitium (interstitial macrophages), as well as freely circulating T cells and NK cells. Immune cells are never observed within the normal seminiferous epithelium in the absence of inflammation or damage.

Most spermatogenic cells are immunogenic (i.e. not tolerised), and can be seen as foreign by the immune cells, so interaction between these cell types needs to be tightly regulated. The blood-testis barrier, more specifically the highly modified tight junctions between adjacent Sertoli cells, effectively isolates the later spermatocytes, spermatids and spermatozoa from immune cells and their secretions. However, the early spermatocytes and spermatogonia can also be immunogenic, and the blood-testis barrier is incomplete

or absent in the region of the rete testis. Moreover, experimental studies have shown that foreign grafts into the interstitial space, outside the blood-testis barrier, are also protected, an observation that was called "immune privilege". Consequently, active regulatory mechanisms are required to prevent immune activation and inflammatory responses from occurring: these are immunosuppression and Treg cell-mediated peripheral tolerance.

Immunosuppression involves locally produced factors that inhibit macrophage and lymphocyte activity, including androgens and other immunoregulatory lipids, lymphotoxic molecules, such as Fas ligand (CD95L), complement and protease inhibitors, and immunosuppressive cytokines, including interleukin-10, transforming growth factor-β and activin A. These factors are produced by the Sertoli cells, Leydig cells and resident macrophages. Sertoli cells and testicular macrophages are inherently immunosuppressive in vitro and can be used to support graft survival in transplantation experiments (Chapter 7). While spermatogenic cells are confined to the seminiferous epithelium, spermatogenic cell antigens constantly leak from the seminiferous tubules, where they are taken up by the resident macrophages, and presented to circulating Treg cells, thereby maintaining antigen-specific Treg-dependent physiological tolerance against the spermatogenic cells.

Immunoregulation in the efferent ducts, epididymis and vas deferens

The local immune environment in the post-testicular tract is quite different from that of the testis. Although the typical epithelial tight junctions are present, there is no complex structure analogous to the blood-testis barrier between Sertoli cells in the duct epithelium. Furthermore, evidence for extended graft survival is lacking. Most strikingly, numerous intraepithelial mononuclear phagocytes are found in the efferent ducts, initial segment and caput epididymidis. These cells appear to physically interact with the sperm exiting from the testis, and are believed to be important for maintaining peripheral tolerance towards the sperm. In the corpus and cauda of the epididymis, these intraepithelial cells are less prominent, and resident interstitial macrophages are more frequently observed. These differences in the distribution of immune cells, as well as differences in the vascularity of the regions, appear to be responsible for the very different responses of the caput and cauda regions of the epididymis to immune activation and inflammation

(Fig. 2). In experimental autoimmune and infection models in rodents, extensive inflammatory and fibrotic responses are largely confined to the cauda region, which can lead to permanent damage to the epididymal duct. By contrast, the caput epididymis is largely unaffected by these stimuli. These regional differences are associated with different sperm antibody responses in different regions of the male reproductive tract as well. Sperm antibody formation in men is more frequently associated with damage or congenital absence of the more distal regions of the epididymis and the vas deferens, in comparison with the more proximal regions of the epididymis.

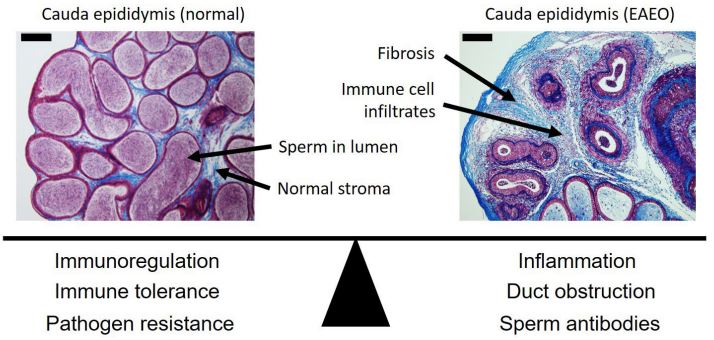

Figure 2: The balancing act of the interaction between the immune system and male reproduction. Under normal physiological conditions, immunoregulation and peripheral tolerance co-exist with endogenous pathogen resistance mechanisms. Left side: micrograph shows a normal mouse cauda epididymis with abundant sperm in the duct lumen and a relatively low proportion of stromal tissue. Right side: micrograph shows a mouse cauda epididymis damaged by experimental autoimmune epididymo-orchitis (EAEO), displaying few sperm, epithelial disruption, extensive fibrosis and immune cell infiltrates. Bar = 200 μm

Physiological role of the immune system in male reproduction

In addition to providing vital protection against reproductive tract infections, the immune system plays a key role in the development and ongoing function of the male reproductive tract. Macrophages

are important for the normal development of the testis, regulation of the spermatogonial niche, maturation and proliferation of the Leydig cells and even steroidogenesis in the adult testis. Genetic ablation of macrophages throughout the body results in male fertility defects in mice, highlighting the importance of these cells in normal reproductive function.

Paradoxically, inflammatory networks have been shown to play a role in the cycle of the seminiferous epithelium. Several inflammatory and immunoregulatory mediators produced by the Sertoli cells, including interleukin-1 and activin A, are involved in the regulation of spermatogonial differentiation and proliferation, meiotic progression and regulation of the blood-testis barrier during spermatocyte transition into the adluminal compartment. This appears to involve inflammatory responses of the Sertoli cells, potentially triggered by the residual cytoplasm from released spermatozoa.

Clinical issues and pathophysiology

While autoimmune disease is rare in the male reproductive organs, sperm autoimmunity can occur when mechanisms of self-tolerance are disrupted or deficient. Physical damage to the male reproductive tract that may occur due to blunt trauma, testicular biopsy, or vasectomy can lead to the formation of sperm antibodies. This results in sperm agglutination and inhibition of sperm motility, as well as blocking surface receptors crucial for fertilization of an oocyte. Immune system involvement in male infertility is highlighted by the fact that testicular biopsies from infertile men frequently contain immune cell infiltrates. T cells are commonly found in biopsies from men with hypospermatogenesis, and immune infiltrates, characterized by T cells, B cells and dendritic cells, are a predominant feature of testicular cancer. Although the clinical significance and consequences of autoimmune inflammation of the testis and epididymis in humans can be difficult to assess, experimental rodent models of autoimmune orchitis and epididymitis show that inflammatory and fibrotic damage in these organs is associated with sperm antibody formation and infertility.

Infections of the male reproductive tract and the resulting inflammation can severely impair fertility, causing damage and fibrosis, both of which can lead to loss of the seminiferous epithelium, and obstruction of the epididymal duct and vas. The steroidogenic Leydig cells are usually affected, resulting in reduced

testosterone levels. In addition, inflammation is also believed to be responsible for ageing/damaged Leydig cells and hypogonadism in older men. Animal studies provide evidentiary support for the use of concurrent anti-inflammatory therapy combined with anti-microbials in treating infectious diseases of the male reproductive system, in order to minimise damage arising from excessive inflammation.

The testis is a common target for viruses.. Many viruses, including mumps, herpes simplex virus-2, hepatitis B and HIV can damage reproductive function in the male. Moreover, viruses can remain sequestered in the testis, due to its immune-privileged environment. This can be a major public health concern, since certain emerging viruses, such as Zika, can be shed in semen of asymptomatic men for up to a year.

In contrast to the testis, the epididymis is primarily the target of infection for ascending bacterial pathogens. In fact, acute epididymitis is one of the most frequent medical presentations in the urological clinic, and is on the rise globally. Although empirical antimicrobial therapy eliminates the pathogen, up to 40% of these patients develop oligozoospermia or azoospermia. Animal studies have shown that the fibrotic damage and obstruction that develops following epididymitis is mostly a consequence of the host immune system response and uncontrolled inflammation.

Prostatitis is most commonly due to ascending urogenital infections, although transurethral manipulation procedures including urethral catheterization or transrectal prostate biopsy can also lead to inflammation of the prostate. While acute bacterial prostatitis responds promptly to antimicrobial therapy, chronic prostatitis may result in chronic pelvic pain. Urethritis is commonly caused by *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium* and Herpes simplex virus, all which are sexually-transmitted. If left untreated, urethritis can develop into ascending infections of the reproductive organs, affecting fertility.

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Chapter 20
What is the prostate and what are its functions?

Gail 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 seminal fluid. The accessory sex glands consist of the prostate gland, the paired seminal vesicles, located at the base of the bladder, and the bulbourethral glands (a.k.a. Cowper's gland) directly connected to the urethra. Collectively, the main function of the sex accessory tissues is to create the seminal plasma, the medium in which sperm are delivered in the ejaculate. The sex accessory tissues produce high concentrations of prostaglandins, fructose, citric acid, polyamines, zinc, and enzymes such as proteases and acid phosphatases. 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.

While seminal plasma substances are not essential for fertilization of the egg by mature sperm, 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 bulbourethral gland is released prior to ejaculation to lubricate the urethra. When ejaculation begins, a sperm-rich fraction is expelled first 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 5% 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 (Fig. 1). In its center, the urethra makes a 70º 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 and empty into the urethra at the level of the verumontanum. Below this site, 20-30 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% of the glandular volume. The peripheral zone surrounds the central zone and extends downward to the prostate apex, comprising 70% of prostatic volume. The tubuloaveolar glands in these regions empty into the prostatic urethra via the aforementioned 20-30 ductules within the 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 proximal urethra is surrounded by a smooth muscle sphincter, the preprostatic sphincter, which contracts during ejaculation preventing retrograde flow of semen into the bladder. It is noteworthy that benign prostatic hyperplasia (BPH) develops in the transition zone surrounding the proximal urethra whereas most prostate cancers develop within the peripheral zone.

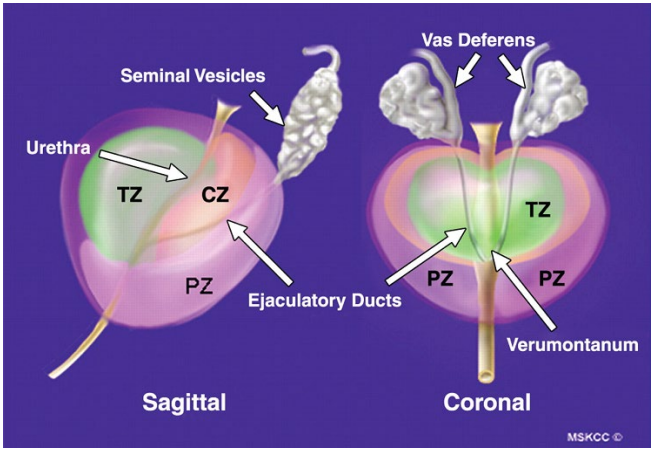

Figure 1. Schematic representation of the prostate gland. PZ: Peripheral zone. TZ: Transition zone. CZ: Central zone. (Reprinted with permission from Currin S. et al, Am J Roentgenol 2007; 188: 3737).

Prostate growth control

Prostate development, function and homeostasis throughout life are entirely dependent on androgens. The primary source is testosterone produced by the testes although adrenal androgens can also contribute to prostate health. Of note, the prostate gland has high expression of reductase enzymes that rapidly and irreversibly convert testosterone to dihydrotestosterone (DHT). Since DHT has a higher affinity for the androgen receptor, this allows for greater androgenic action within the prostate gland without over-androgenizing other peripheral organs that utilize testosterone.

Diseases of the prostate gland

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. A brief overview of these conditions is presented below and some will be discussed in greater detail in chapters of their own (Chapters 56-59). 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% by 80 years of age. 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 surgery (e.g. TURP) as well as medical management with alpha-adrenergic blockers and/or 5α–reductase inhibitors.

Prostate cancer, an adenocarcinoma, is the most common non-cutaneous 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. African men have the highest mortality rate due to prostate cancer in the world. 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 are typically age and stage-dependent and include watchful waiting, active surveillance, surgery (prostatectomy) or radiation for early stage disease (i.e. confined to prostate). Side effects of surgical and radiation therapy can include incontinence and impotence. When prostate cancer reemerges or is diagnosed at the metastatic stage, androgen-deprivation therapy (ADT) without or with chemotherapy (e.g. taxanes) is the first line of therapy for this later-stage disease. Like the prostate gland itself, prostate cancer initially depends on androgens to grow. Unfortunately, as the disease progresses, particularly with extended ADT duration, it becomes independent of androgens with no curative treatments available. With second and third generation ADT modalities, ~30% of metastatic disease will emerge as neuroendocrine (small cell) prostate cancer with no available treatments at present. Side effects associated with ADT include sexual dysfunction, infertility and muscle and bone wasting. In the past decade, several new treatment approaches have emerged for advanced prostate cancer therapy including immunotherapy, PARP inhibitors, immune checkpoint blockade, advanced radiation approaches (^{177}Lu-PSMA-617; radium-223) and novel approaches continuously under development. The goal is to prolong life and maintain a high quality of life so that men continue to live with the disease and not die from it.

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Chapter 21
What are the seminal vesicles?
Development, secretions and functions

Paul S. Cooke and William H. Walker

Seminal vesicle structure and function

The seminal vesicles, along with the prostate and bulbourethral glands, are accessory sex glands that are found only in males (Fig. 1). These bilateral glands are comma-shaped structures located between the bladder and the rectum that in men are each 5-7 cm long when uncoiled, with a volume of approximately 13 ml. Each seminal vesicle consists of one long highly infolded sac lined with a single cell layer of pseudostratified secretory epithelium atop a discontinuous layer of basal cells and a thick outer layer of smooth muscle cells innervated by sacral nerves S2-S4 (Fig. 2). The extensive infoldings of the seminal vesicle epithelium maximize the epithelial surface area and secretory capacity of the organ.

The main function of the seminal vesicles is to produce a complex fluid that is incorporated into the ejaculate and facilitates various aspects of the subsequent process of fertilization. Seminal vesicle secretions are stored within the gland as a viscous fluid until they are expelled at the time of orgasm. The seminal vesicle fluid is expelled through a single duct at the time of orgasm, where it mixes with other components of the developing ejaculate, as described in detail below.

Role of androgen signaling in seminal vesicle development and function

Seminal vesicles are derived from the Wolffian ducts of the male fetus, which are paired embryonic structures that also give rise to the epididymis and ductus deferens. The seminal vesicles originate from the most caudal portion of the Wolffian ducts, near where these bilateral ducts juncture with the fetal urogenital sinus. Initial masculinization of the fetal reproductive tract and the differentiation of the epididymis, ductus deferens, seminal vesicles, prostate and bulbourethral glands in the male occurs in response to androgens

secreted by fetal Leydig cells in the testis. Mammalian embryos are initially ambisexual, and Wolffian ducts initially develop in the female embryo as well. However, in the absence of exposure to amounts of androgen sufficient to cause the stabilization and development of the Wolffian ducts, such as occurs in the male embryo, the female Wolffian ducts regress.

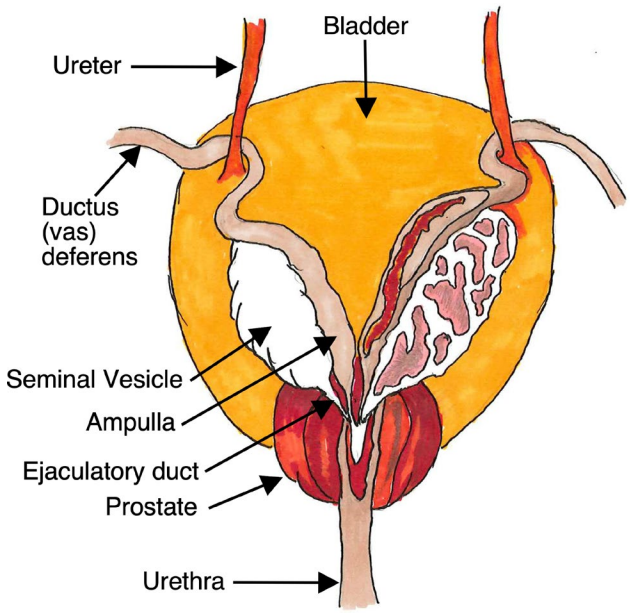

Figure 1. Posterior view of human urogenital organs including seminal vesicles, bladder, urethra, prostate and ejaculatory ducts. Intact organs are shown on the left. Internal structures of the organs and the formation of the ejaculatory duct are shown in the central region and on the right.

Following their fetal differentiation, the seminal vesicles and the other accessory sex organs in the male remain immature until the time of puberty. This pre-pubertal interval in various species is highly variable, ranging from a few weeks in mice to over a decade

in humans. Increasing androgen concentrations during puberty induce rapid growth, morphogenesis and secretory function of the seminal vesicles. These glands are obligatarily dependent on androgen not only for pubertal development but also for maintenance of their adult function and secretory capabilities. Loss of androgen signaling by castration or other treatments that block normal androgen signaling (e.g., anti-androgens) induces a striking and rapid involution of these structures and abolishes their secretory activity at any age in the adult mammal.

Androgen receptors (ARs) are essential for seminal vesicle organogenesis, as demonstrated by studies showing that transgenic mice lacking AR do not develop seminal vesicles. The AR present in the Wolffian duct, as well as other fetal structures such as the urogenital sinus that forms other accessory glands, function as ligand-activated transcription factors that stimulate the initial development and morphogenesis of the seminal vesicles.

Development of urogenital organs such as seminal vesicles and prostate involves reciprocal mesenchymal-epithelial interactions. Androgen receptors are present initially in the mesenchyme, but not the epithelium, of fetal organs such as Wolffian ducts and urogenital sinus. Androgen acts to stimulate seminal vesicle epithelial development indirectly through AR in the mesenchyme, and then the mesenchymal cells drive epithelial development and proliferation through mechanisms that still remain unclear.

Adult seminal vesicles express AR and estrogen receptors (ESR1, ESR2) in stromal, smooth muscle and epithelial cells, and various transgenic models have been developed to demonstrate unique roles of AR in various cell types such as the seminal vesicle smooth muscle. For example, seminal vesicles lacking smooth muscle AR were 55% smaller than normal. Proximal epithelial cells were low and cuboidal with little cytoplasm indicating that testosterone-derived signals from smooth muscle cells are important for maintaining normal epithelial cell function including secretion. In adults, the epithelial layer was less folded, and the stromal smooth muscle layer depth was reduced about 50% and disorganized. The numbers of copulatory plugs were similar to normal 4 h after coitus but were smaller with a soft and fibrous consistency that likely is responsible for the 70% decrease in plug-positive females when assessed the morning after coitus. In vivo fertilization was reduced by more than 60% and in vitro fertilization was reduced at low sperm concentrations, perhaps due to a mild defect in sperm binding to the zona pellucida.

Androgen signaling in smooth muscle may also serve to limit epithelial cell proliferation, as the number of epithelial cells undergoing mitosis in mice lacking AR in seminal vesicle smooth muscle cells was increased 3-fold, resulting in hyperplasia. Elevating estradiol (E_2) while keeping testosterone levels constant also increased epithelial cell proliferation by more than 2-fold. Increased E_2 did not alter epithelial cell height suggesting no regulation of epithelial cell differentiation, but the depth of the smooth muscle layer was increased indicating that E_2 supports smooth muscle proliferation.

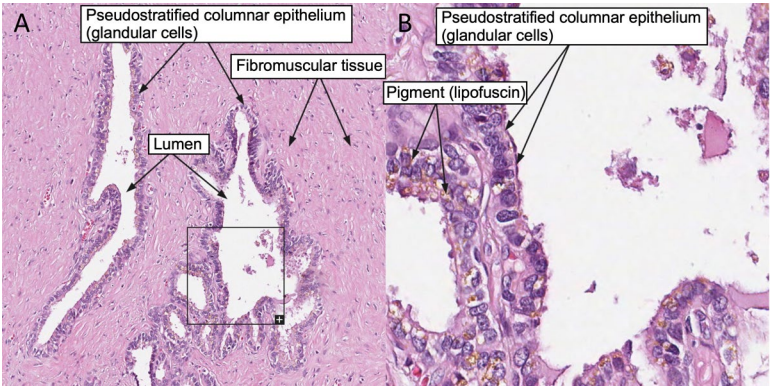

Figure 2. A) low power view of a cross section of seminal vesicle showing fibromuscular tissue surrounding pseudostratified columnar epithelia and **B)** a higher magnification view of the region within the box in A showing the single layer of epithelial cells. From Pontén F, Jirström K, Uhlen M. The Human Protein Atlas-a tool for pathology. J Pathol. 2008 216(4):387-93.

Release of seminal vesicle fluid into the ejaculate at the time of orgasm

The seminal vesicle mucosa is surrounded by extensive tunics of smooth muscle that can propel the seminal vesicle secretion into the urethra at the time of ejaculation, and release of seminal vesicle fluid at the time of ejaculation is partially due to the contraction of the smooth muscle elements in the seminal vesicles themselves. In addition, contraction of a number of striated muscles in the urogenital region also facilitate the expulsion of seminal vesicle fluid

as well as that from other glands such as the prostate and is critical for progression of the ejaculate through the male reproductive tract.

Release of seminal vesicle fluid is coordinated with that of the other components of the ejaculate to insure near-simultaneous expulsion of all of the components that comprise the seminal fluid. Sympathetic discharge at orgasm causes vigorous contraction of the seminal vesicles and the rapid movement of the seminal vesicle fluid through the excretory duct of the seminal vesicles. Simultaneously, sperm and associated fluid originating from the testis and epididymis are conveyed down the ductus deferens. The seminal vesicle excretory duct merges with the distal ductus deferens to form the ejaculatory duct that empties into the urethra where it passes through the prostate (Fig. 1). Secretions from the prostate are then added to the developing ejaculate, and finally bulbourethral gland secretions are added at the base of the penis to complete the ejaculate that then passes into the female reproductive tract.

Although many organs contribute components to the final ejaculate, seminal vesicles provide a major portion of the ejaculate volume. The exact amount of the semen volume that originates from the seminal vesicles varies in different animals, but studies using a variety of species and various methods of measuring the contribution of the seminal vesicle fluid to the ejaculate suggest that seminal vesicle secretion is the major constituent of the ejaculate and makes up more than half of the ejaculate volume in humans as well as the other animals studied.

Role of the seminal vesicle in clotting of seminal fluid

In species such as rats, mice and some non-human primates, the seminal fluid forms a copulatory plug following its deposition in the vagina. These copulatory plugs in species such as rodents inhibit leakage of seminal fluid from the vagina, and the plugs in rodents are gelatinous and durable. Copulatory plugs in rodents can be detected many hours after a successful mating, and function as a reliable record of successful copulation in these species.

Copulatory plugs are formed in the female reproductive tract due to clotting proteins derived from the seminal vesicle fluid associating with enzymes from the prostate. Human semenogelin I and II (corresponding to seminal vesicle secretion proteins SVS2-SVS6 in mice), the main components of the fibrous coagulum, are secreted from seminal vesicles. Like rodent semen, human semen

also coagulates after ejaculation. However, in striking contrast to rodents, which form long-lasting and resilient copulatory plugs, following clotting human semen will liquify within a few minutes due to cleavage of semenogelin by prostate-specific antigen, a chymotrypsin-like prostatic protease. Coagulation allows sperm to be coated with nutrients and factors that contribute to fertilization. Subsequent liquefaction permits sperm to swim into the cervix and uterus (Chapter 25).

Seminal vesicle fluid components and activities

Seminal vesicle fluid is a slightly alkaline secretion that is highly viscous and contains fructose, which serves as an immediate energy substrate for ejaculated sperm to support their motility. Low levels of fructose in semen are an indicator of seminal vesicle obstruction or dysfunction. Seminal vesicles also produce citric acid, which modulates pH and metabolism. In addition, the seminal vesicle secretes semenogelin/SVS proteins, metallothionein-1 (Mt-1), and transglutaminase-4 (TGM4). Aquaporins present in the apical and basolateral membranes of seminal vesicle secretory cells regulate water transport contributing to high concentrations of proteins (up to 200 mg/ml) and the viscosity of seminal vesicle fluid. Expression of aquaporins in seminal vesicles (and prostate) is dependent on testosterone as castration decreases the levels of at least 5 aquaporin proteins in rats.

Seminal vesicle-derived prostaglandins in semen increase female genital tract smooth muscle contractions to aid in sperm transport. Seminal vesicles also secrete various ions, enzymes, and the proteins involved in the clotting process of the semen after ejaculation. Reactive oxygen species (ROS) scavengers including superoxide dismutase, catalase glutathione peroxidase/reductase, ascorbic acid, uric acid and thiols are produced to counteract elevated levels of ROS and oxidative stress that are associated with decreased sperm motility and sperm-oocyte fusion. Seminal vesicle secretions facilitate fertilization, and a number of studies have shown that the fertility of rodents with their seminal vesicles removed is reduced, but not eliminated.

Seminal vesicles are somewhat unique among the accessory sex organs in that they are species variable. Some groups of animals such as carnivores, e.g., cats, and cetaceans, e.g., whales, lack seminal vesicles. The data in species such as rodents showing diminished fertility in the absence of the seminal vesicles suggests that the lack

of the seminal vesicles would be expected to inhibit fertility, but these species that have lost their seminal vesicles during their evolution may have other methods of compensating for the loss of seminal vesicle fluid and its normal supporting role in fertilization.

Clinical relevance of the seminal vesicles

The accessory sex organs in general have important medical significance due to the prevalence of two major prostate diseases in men that are of immense clinical importance, benign prostatic hyperplasia (BPH), which afflicts 80% of men 70 years of age and older, and prostatic cancer, which is the second most common neoplasia in men. Although BPH also occurs in the dog as well as human, this disease does not naturally occur in other species. The reasons for this are unknown.

Despite the extensive similarities between the prostate and seminal vesicles in terms of anatomical location, secretory function and hormonal regulation, clinical problems involving the seminal vesicles are rare. Seminal vesiculitis can occur as a result of bacterial infections arising from surgery or sexually transmitted diseases, but the incidence of this is very low. Seminal seminal vesicle cancer is exceedingly rare, with only about 60 cases described in the literature, and no other common clinically significant problems related to seminal vesicles occur. The reason that these two organs, which share extensive developmental and functional similarities, are totally different in terms of their susceptibility to pathological changes has been speculated on for decades but remains unknown despite the clinical importance of the question.

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Chapter 22
How does semen analysis assist in understanding the reproductive status of the male?

Christina Wang

What composes semen?

The ejaculate consists of spermatozoa (sperm) in a mixture of secretions mainly from the prostate gland (Chapter 20) and seminal vesicles (Chapter 21) with small contributions from the epididymides (Chapter 18). 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, about 90% of volume of the seminal fluid is composed of secretions of from the prostate and seminal vesicles. There is some evidence that the total volume of the ejaculate collected in a clinic may be lower than that during sexual intercourse. As the volume of the seminal fluid may be quite variable, the total number of spermatozoa in the ejaculate may be a more important parameter than the sperm concentration in the ejaculate.

The structure of the human spermatozoon is shown in Fig. 1. Enclosed in a plasma membrane, the spermatozoon consists of the head which has the nucleus (chromatin containing the genetic material and epigenetic elements) covered by the acrosome. The flagellum (tail) has a mid-piece, principal piece, and end piece. The principal piece contains nine microtubule doublets plus a central doublet forming the axoneme which is surrounded by outer dense fibers enclosed by the fibrous sheath. The midpiece contains mitochondria wrapped around dense fibers and axoneme. The end piece has lost the axoneme structure as well as the outer dense fibers and fibrous sheath. The flagellum 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.

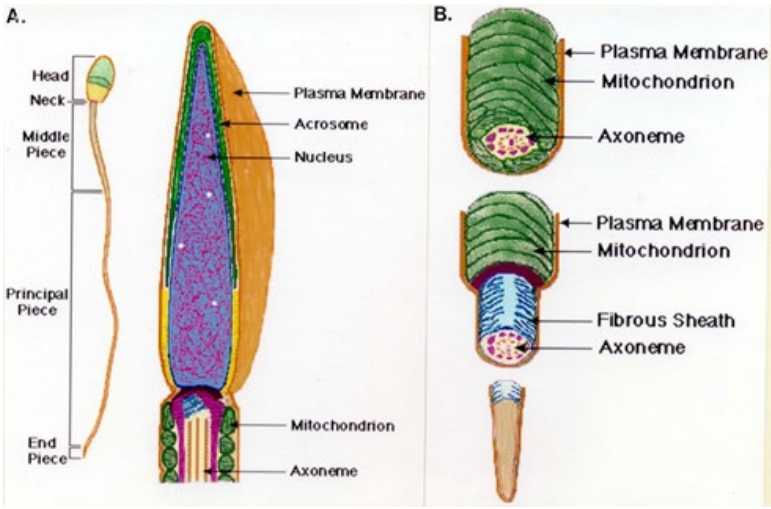

Figure 1. A) Major elements of a human spermatozoon that are common to mammalian species. **B**) Middle piece (top), principal piece (middle), and end piece (bottom) of a spermatozoon viewed in cross-section.

Why is semen analysis important?

Semen analysis provides insights to the reproductive health of the male. Laboratory examination of semen is used for the assessment of:

1. Male reproductive function;
2. Fertility potential of the male and may assist in choosing the appropriate treatment;
3. Efficacy of male contraceptive methods; and
4. Exposure to environmental pollution, drug, irradiation, or other toxic chemicals on reproduction in animals and men in epidemiological studies.

How is semen analyzed?

The World Health Organization developed a laboratory manual to provide a guide regarding acceptable methods for semen analysis in man. The goal of this manual is to contribute to improvement of assessment of male reproductive function and to standardize semen analyses so that results are comparable throughout the world. Most of the techniques can be applied to other species. In rodents, the

ejaculated semen forms a coagulum and does not liquefy, thus sperm parameters are assessed by examining spermatozoa in the cauda (tail region) of the epididymis. In rabbits, semen can by 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 the 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 basic parameters that are usually assessed are:

- 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.

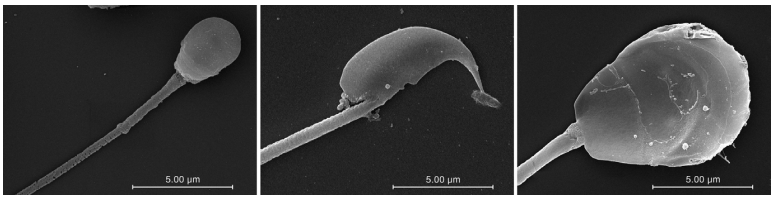

Figure 2. Scanning electron micrographs of human (left), mouse (middle), and guinea pig (right). Spermatozoa. Courtesy of G. Hunnicutt, The Population Council

What is a "normal" semen analysis in fertile man?

There are fluctuations in semen parameters from day to day in men and usually two semen samples are required to diagnose that the semen quality is below the distribution limits of fertile men. The World Health Organization in 2010 determined distribution of semen parameter from fertile men with a time to pregnancy of < 12 months and used the 5th percentile as the lower distribution limits. A follow-on study in 2021 included data from the 2010 study and added new data that resulted 3589 fertile men (with a time to pregnancy of < 12 month, defined period of abstinence between 2 to 7 days,) living in five continents to provide distribution limits of semen and sperm variables of adult men. In the latest edition of the World Health Organization laboratory manual, the lower 5th percentile for semen parameters in "fertile" adult men are:

- semen volume 1.4 mL;
- sperm concentration 16 million/ml;
- total sperm number per ejaculate 39 million;
- sperm progressive motility 30%, total sperm motility 42%;
- sperm vitality 54 % alive;
- spermatozoa with normal morphology 4 % (using strict criteria that exclude any spermatozoa with even a mild abnormality).

It is important to note that there is substantial overlap of sperm parameters from fertile and infertile men. These distribution limits are from fertile men, values above or below the 5th percentile do not provide a clear boundary between fertility and infertility.

What other tests on semen/sperm may be useful to assess male reproductive function?

The ability of the live sperm tail to swell under hypo-osmotic conditions is a test of sperm membrane integrity and sperm vitality. Special staining may reveal white blood cells in semen samples; this is suggestive of an infection. Semen biochemistry is indicated when accessory organs problems are suspected, e.g., semen fructose is low in men with congenital absence of the vas deference and seminal vesicles.

Other tests to assess sperm quality may be useful for diagnosis of fertile and infertile men and for research purposes but not recommended for routine analyses until clear predictive values are defined. Multiparametric sperm morphology assessment compiles data obtained at routine analyses into an index that may provide

increased accuracy and consistency of sperm morphology. Acrosome reaction, which occurs near the oocyte in the fallopian tubules, is a process where the acrosomal membrane fuses to expose the sperm head for fertilization of the oocyte (Chapter 25). The percentage of induced acrosome reacted sperm has been associated with fertilization rate. Sperm DNA quality and fragmentation are associated with miscarriage rates and in vitro fertilization outcomes and are used is studies on exposure to testicular toxicants (Chapter 23 and 42). The use of DNA fragmentation in assessment of male infertility is controversial. Computer aided assessment of sperm motility characteristics are not very useful for clinical diagnosis but is frequently used in research and epidemiological studies. The ability of sperm to exhibit hyperactivated motility is essential for sperm to migrate through the female reproductive tract and penetrate the zona pellucida. Computer aided sperm analyses is useful in the characterization of "hyperactivated motility" and may provide some insight on the signaling systems necessary for fertilization (Chapter 25). New developments allow men to check the presence of motile sperm by using apps on a mobile phone, but current methods lack accuracy when compared to laboratory-based methods. There are also at-home use kits for estimating the sperm concentration using immunoprecipitation. Evolving technology include use of genomic and epigenomic testing of sperm to define the causes of abnormal sperm head and flagella.

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

Semen analysis is 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 environmental factors and toxicants on male reproductive function (Chapters 41, 42). Suppression of the number, motility, or function of spermatozoa in the ejaculate to very low levels is the goal of male contraceptive development (Chapter 30, 31). However, in clinical medicine semen analyses is used primarily for the diagnosis and treatment of male infertility (Chapters 33, 35). The diagnosis for male infertility is dependent, in large part, on the analysis of semen samples. Most men diagnosed with infertility 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 show grossly low values, male factor infertility is diagnosed. The concentration of spermatozoa and their quality 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 sperm concentration, total progressive motility, normal morphology, and hypoosmotic swelling test (detects viable sperm) correctly identify 84.1% of 111 fertile men and 88.1% of 109 infertile men. In association studies, time to pregnancy is best predicted by combination of sperm concentration, motility, and morphology. Thus, from these prospective clinical studies, sperm concentration, total sperm count, sperm progressive motility, 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 probability of conception. How useful are additional functional tests in predicting fertility has not been studied in prospective large scale clinical studies.

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Chapter 23
How is sperm chromatin structure quality assessed? What is the value of doing such assessments?

Bernard 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 2021. As discussed in Chapter 22, these parameters are useful in population studies; when parameters such as total sperm number, concentration or sperm motility are very high or very low it is clear that there is a strong correlation with fertility outcome. However, the semen sample parameters of many individuals fall well below the WHO reference values, yet they can father children; on the other hand, men with semen parameters well above the WHO reference values may experience infertility. Thus, a very large proportion of unexplained (idiopathic) infertility may be due to factors other than those determined in standard semen analysis.

The parameters used in routine semen analysis are not designed to assess the "quality" of the spermatozoa, other than their ability to swim and "appear" normal. What matters for successful fertility is not just getting a single spermatozoon to an oocyte but also delivering its 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.

Although the most recent WHO manual does not include assessment of sperm chromatin integrity as part of routine semen analyses, it does include an extensive section on the value of extended examination of semen and concludes that: "Clinically, there is growing awareness that chromosomal anomalies and gene

mutations underlie a diverse spectrum of male infertility that underlie many of the anomalies seen in a semen analysis." (p.83, WHO Manual 2021).

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, histone modifications), how it is packaged with non-coding RNAs, associated with the nuclear matrix, and condensed with protamine cross-links and histones.

Starting more than fifty 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 thanks to 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, florescence and confocal microscopy, flow cytometry, real time PCR and high throughput screening. There are a number of ways of groupings 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

TUNEL Assay. The quantity of open 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 a flow-cytometry assay.

Comet Assay. This electrophoresis-based assay evaluates DNA strand breaks in a single spermatozoon. 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 the assay is run at basic or neutral pH, the Comet Assay will assess single or double strand breaks, respectively. Although a novel high-throughput assay (HT-COMET) has been developed, this very sensitive procedure requires the use of expensive equipment. While providing a powerful index of DNA

damage, the Comet assay is time consuming and the results are variable among labs.

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 complimentary 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 thousands of sperm manually for accurate results renders this method still primarily a research tool.

Assays used to evaluate sperm chromatin conformation and proteins

Acridine Orange Flow Cytometry or SCSA® Assay. This commonly used flow-cytometry based assay assesses the susceptibility of sperm chromatin to denaturation in acid and detergent as determined by acridine orange binding to double- or single-stranded DNA; this dye gives a green fluorescence for unfragmented DNA and a red fluorescence for fragmented DNA. After denaturation, measurement of fluorescence at both wavelengths assesses the percentage of fragmented DNA (DNA fragmentation index: DFI). This assay allows for rapid assessment of a large number of cells; it may also be run as a "slide assay", where the color reading is made on microscope slides, but where only a few cells are monitored.

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.

CMA3 Fluorometric Assay. This assay indirectly measures the amounts of protamine present, thus reflecting the extent of chromatin condensation. 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 (fluorescence-activated cell sorting) assay.

Monobromobimane (mBBr) Thiol Labeling Assay. The objective of this assay is to determine the amount of free sulphydryl groups in spermatozoa. Less mature sperm have more free sulphydryls, 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.

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 and opens the possibility of new criteria to be assessed when assessing sperm quality.

Assays used to evaluate the epigenetic status of spermatozoa

DNA Methylation. DNA methylation of cytosine residues is one of the major epigenetic marks established during spermatogenesis. Both single site and genome wide methylome studies have revealed alterations in the methylome under various conditions, including smoking and advanced age. Indeed, the sperm methylome can be used to accurately predict a man's age. The identification of specific changes in DNA methylation patterns with specific causes of infertility is under investigation (Chapter 13).

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 in their numerous marks, e.g.,

acetylation, methylation, sumoylation, appear to affect the functions of spermatozoa (Chapter 14).

Long and Small Non-coding RNAs. The third major pillar of epigenetic regulation is the expression and function of a wide range of non-coding RNAs that include both long (mRNA, lncRNA, and circRNA) as well as short, noncoding RNAs (miRNAs, piRNAs, tsRNAs, and snRNAs) (Chapter 15). The exact functions of these various RNAs are actively being pursued. However, there is clear evidence that some of them play an essential role in the development of 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.

Conclusions

In an effort to help standardize some of these sperm chromatin structure assays, the WHO 2021 manual has provided protocols for the TUNEL assay, sperm chromatin dispersion test, acridine orange flow cytometry assay (*SCSA*®), and for fluorescence in situ hybridization.

No consensus has yet emerged regarding the value of any one or a group of these tests in assessing the fertility of an individual, but their usefulness in certain circumstances such as recurrent miscarriages, or unexplained fertility is now appreciated (Chapter 32). Furthermore, the growing understanding of the complexity of what is in a sperm nucleus and how it is packaged, as well as the increased precision and accuracy of newer tests, makes it likely that new clinically useful tools to accurately assess the quality of spermatozoa will be used clinically in appropriate subgroups of patients.

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Chapter 24
What is sperm banking? When and how is it (or should it be) used in humans? Animals?

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

Susan A. Rothmann, William C. Baird III, Matthew C. Hudnall, William C. Baird IV

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. However, the development of many sperm banking applications did not begin until after 1950, following the discoveries that glycerol can act as a cryoprotectant and ultra-low temperature liquid gases, especially liquid nitrogen, were suitable for freezing and long-term 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 exposures, surgery, 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 distant locations.

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, -196oC, 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

Clinically, sperm banking encompasses donor sperm 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) Regulations. 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 also require separate licensure with inspection. Many other countries also have regulations about sperm banking.

The most common use of donor sperm is treatment of infertility caused by absent or defective sperm, and for individuals or couples without a sperm producing partner, especially single women and women only couples. In a 1987 survey, the United States Office of

Technology Assessment estimated that 30,000 births resulted from artificial insemination with donor sperm, with approximately 11,000 physicians providing the treatment to about 86,000 women. A more recent study estimates that roughly half a million women were estimated to have used donor or mixed (husband and donor) sperm. However the U.S. does not maintain records on the usage of donor sperm, thus these numbers are extrapolated through surveys.

The practice of "quarantining" 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 any single donor within a limited geographic area with resulting risk of consanguinity in future generations. Population statistics can estimate this risk; generally, sperm from a single man should be limited to 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 it depends on the live birthrate, number of inhabitants and ethnic composition.

Usually, sperm banks attempt to package donor sperm in plastic vials or straws containing at least 10 million motile sperm post-thaw; this has been accepted as the minimum adequate insemination dose. Frozen- thawed sperm have shorter longevity and less ability to penetrate cervical mucus than fresh sperm, making the route and timing of insemination critically important in achieving a successful pregnancy. Using urinary luteinizing hormone (LH) measurement to predict ovulation, and one or two intrauterine inseminations (IUI) 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's 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 such as vasectomy.
3) Anticipated exposure to hazardous environments. Occupational exposure to radiation, pesticides, and chemicals can affect sperm function or genetic integrity. Men engaging in military operations where risk of death or exposure to sperm toxicants exist are also candidates for sperm storage.
4) 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.
5) Gender affirmation surgery and/or hormone replacement therapy (HRT).

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 single sperm injection into the ooplasm (intracytoplasmic sperm injection, ICSI), greatly improve chances of successful pregnancy with limited numbers of sperm. Having many sperm stored is definitely an advantage since it may reduce the need for in vitro fertilization or allow multiple cycles of fertility treatments. However, the desire to bank multiple ejaculates with adequate abstinence periods between collections, must be balanced against the urgency of treatment initiation. Given the increasing number of

sperm banks, reproductive centers and laboratories and overnight mail-in banking services, all men and boys who might have compromised fertility as a consequence of their disease or its treatment should be offered reproductive consultation and the opportunity to cryopreserve 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 and increase genetic diversity.

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 and entrepreneurs in both human and animal applications from the laboratory to the bedside or "barnside".

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Chapter 25
How are the sperm and egg prepared for fertilization and how does fertilization take place?

Janice P. Evans, James A. Foster and Janice L. Bailey

Fertilization is a complex, multi-step process involving sperm and egg maturation, transport, and interactions that culminate in fusion of the two cells and their haploid genomes. This fascinating biological event 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 of meiosis I where it has been arrested since before birth, to an arrest in metaphase of meiosis 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 18), sperm develop the ability to (1) be motile and (2) undergo capacitation (addressed below). Next, upon ejaculation, the sperm are mixed with seminal fluid, which includes buffering components that help sperm survival in the acidic environment of the vagina. Seminal fluid has additional functions in reproduction as well, including providing proteins that attach to the sperm and mediate sperm binding to the oviduct, and signaling factors (e.g., cytokines, prostaglandins, hormones) that induce changes in the female reproductive tract that facilitate fertilization and reproductive success.

During sperm transit through the female reproductive tract, sperm are modified by interactions with secreted fluids, extracellular vesicles, and epithelial cells. In the female tract, sperm undergo capacitation, which are the physiological changes and reorganization of the sperm surface that confer the ability to fertilize an egg. It is notable that the discovery in the 1950s of methods to support sperm capacitation *in vitro* is one of the advances that made

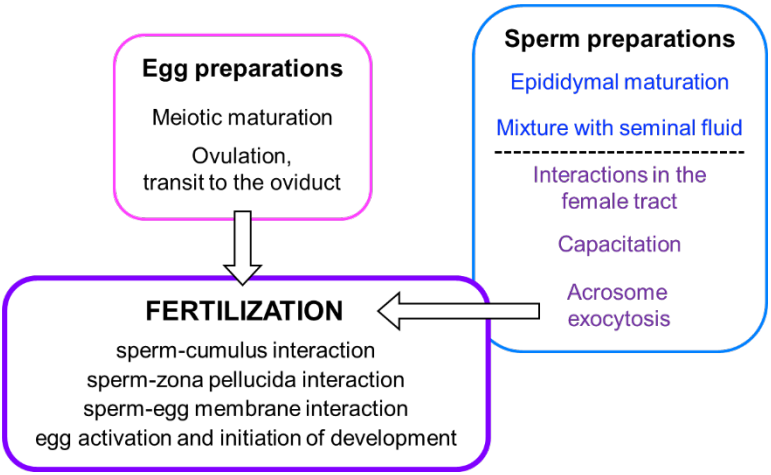

Figure 1. This schematic diagram shows the steps discussed in this chapter, including the egg's preparatory steps (pink box), the sperm's preparatory steps (blue box), and the steps of fertilization itself (purple box). The steps of the sperm's preparations in blue text and above dotted line occur in the male, and the steps in purple text below the dotted line occur in the female. Acrosomal exocytosis, the last step of the sperm's preparatory changes, occurs after capacitation. There are multiple possible triggers for acrosome exocytosis, originally thought to be binding of sperm to the zona pellucida (ZP) during fertilization, but newer data suggest that acrosome exocytosis can occur while the sperm are in the upper isthmus of the oviduct, stimulated by factors such as progesterone and/or mechanical shear force (see main text).

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 acrosomal exocytosis. 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. This change in motility is thought to help sperm bind and transit through the vestments surrounding the egg, which include the cumulus cell layer and the zona pellucida (Fig. 2).

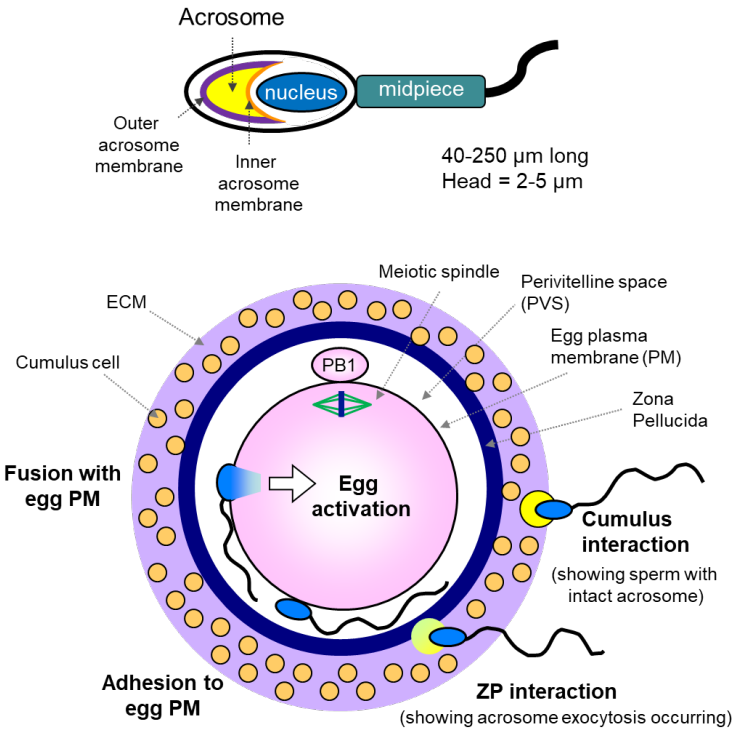

Figure 2. Upper panel: Schematic diagram of a mammalian sperm, indicating the two main domains, the head and the tail. The flagellar tail contains microtubules, and includes the midpiece, which is where the mitochondria are located. The head includes the sperm chromatin in the nucleus (blue), and the acrosome (yellow). Acrosomal exocytosis is achieved by the formation of fusion pores between the outer acrosomal membrane (purple) and plasma membrane (black). After acrosomal exocytosis is complete, the inner acrosomal membrane (orange) is exposed on the surface of the sperm. Lower panel: Schematic diagram showing the structures of the mammalian egg and the steps of fertilization (bold text). This diagram illustrates acrosomal exocytosis occurring with sperm-ZP interaction, although newer data suggest that there are other triggers that can induce acrosomal exocytosis prior to ZP penetration (see main 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 pellucuda 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 activaton. occurs.

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. In other species, sperm can even be stored in specialized sperm storage tubules in the female tract for weeks, months, or years. 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 muscular 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.

Fertilization occurs as a continuous process with several identifiable steps: (1) sperm-cumulus interactions, (2) sperm-zona pellucida interactions, (3) sperm-egg plasma 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 may also be facilitated by hyaluronidases on the sperm surface.

The sperm next interacts with the egg's coat, the zona pellucida (ZP), which is synthesized during oogenesis by the developing egg 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, but sperm-ZP binding is likely mediated by a group of proteins and may depend on the 3D conformation of a protein complex. The ZP supports sperm binding in a species-selective manner but whether and how the ZP induces the sperm to undergo acrosome exocytosis is debatable. Also known as the acrosome reaction, acrosomal exocytosis involves (1) the release of the contents from the acrosome, the large secretory vesicle on the head of the sperm, and (2) additional reorganization of the sperm surface (Fig. 2). The release of the acrosome's contents is accomplished by the formation of numerous fusion pores between the outer acrosome membrane and the head plasma membrane, and is thought to proceed in a sequential manner, with soluble proteins being released more readily than acrosomal matrix proteins that are proteolytically released, at least in part, by acrosin and other

proteases. As acrosomal exocytosis progresses to completion, new arrangements of proteins on the sperm surface are exposed, which render the sperm capable of interacting with the egg plasma membrane. Thus, acrosomal exocytosis is a necessary prerequisite for the next step of fertilization, interaction with the egg plasma membrane (see below). The signal triggering the sperm to undergo acrosomal exocytosis was thought to be binding to a specific ZP component, but recent studies in mice have cast doubt on this model and instead suggest that acrosomal exocytosis is triggered in the upper isthmus prior to reaching the oocyte by other, and perhaps multiple, factors (e.g. progesterone, mechanical shear force). Downstream from this initial trigger, calcium is a key second messenger that induces the sperm to complete acrosomal exocytosis.

Once the sperm has penetrated through the ZP, it reaches the perivitelline space where it gains access to the 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). Studies using mouse knockout models have identified multiple sperm and egg molecules that are critical for sperm-egg membrane interactions. Upon the formation of cytoplasmic continuity between the gametes, a sperm-specific form of phospholipase C (PLCζ) is among the intracellular components introduced into the egg from the sperm. PLCζ plays a crucial role in inducing the egg-to-embryo transition based on studies of mouse knockout models and certain male infertile patients with sperm that are defective in initating embryo development, although some data suggest that compensatory factors may exist in sperm. Once introduced into the egg cytoplasm, PLCζ generates IP3 (inositol triphosphate) from PIP2 (phosphatidylinositol 4,5-bisphosphate). IP3 in turn binds to IP3 receptors on the egg's intracellular Ca2+ stores, the endoplasmic reticulum. This causes release of Ca2+ into the cytosol, and the activation of this Ca2+-dependent signaling pathway induces the egg-to-embryo transition, also known as egg activation. Thus, the egg must be capable of responding to this sperm-borne PLCζ,; interestingly, there may be an egg factor(s) that mediates this responsiveness, as PLCζ induces increased cytosolic calcium in eggs but not in other cell types. 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 summary, sperm and egg maturation involve several highly orchestrated processes that occur in the male and female reproductive tracts, culminating with gamete fusion and triggering the egg-to-embryo transition. Fertilization is a continuous series of processes that result in the delivery of the paternal chromatin to form an embryo and ultimately, competent offspring.

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Chapter 26
What determines biological maleness?
Sex determination, testis formation and development of the male phenotype

Nicolas Pilon and Robert S. Viger

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

In humans, male and female sex phenotypes are the typical outcomes of the development of biological sex. 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 typically an individual with an XY sex chromosome complement, having testes and male external genitalia (penis and scrotum) and internal accessory sex organs (epididymis, vas deferens, prostate, 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 (XO) 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 (typically) be a biological male; if an X bearing sperm cell unites with the egg, the resulting zygote will have an XX sex chromosome complement and will (typically) be a biological female. The correlation of the Y chromosome with biological 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 testes

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 gonads develop from the paired genital ridges, found on the roof of the abdominal cavity of the fetus during organogenesis. The genital ridges initially have the capacity to form either testes or ovaries: they are bipotential. 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 results 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), which 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 cords structures (in the interstitium). Leydig cells produce two crucial hormones in the developing XY fetus: the protein insulin-like 3 (INSL3) and the steroid testosterone. INSL3 initiates the trans-abdominal phase of testicular descent. Testicular descent into the scrotum (inguino-scrotal phase) is then completed through the action of testosterone. Testosterone and its metabolite dihydrotestosterone (DHT) further 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 also masculinizes the developing brain. In the XX fetus, the absence of testosterone (and presence of maternal and fetal estrogens) ensures that the external genitalia develop as a vagina and labia and that internally, the mesonephric duct atrophies. 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 testis. 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 exclusively found in placental mammals and has very poor sequence conservation between species. As a consequence of *SRY* expression, pre-Sertoli cells express the highly conserved *SOX9*. SOX9 appears as strong as SRY for promoting biological maleness, as overexpression of *Sox9* in mice also forces the development of testes in the XX fetus. SOX9 will also turn on expression of the *MIS*/*AMH* gene. The current picture of 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* and Fibroblast Growth Factor 9 (*FGF9*) expression, and the female pathway which responds to a number of pro-female factors that include the signaling molecules R-Spondin Family Member 1 (RSPO1) and *Wingless*-related MMTV integration site 4 (WNT4), and the transcription factor Forkhead Box L2 (FOXL2). In a typical XY genital ridge, the presence and expression of the *SRY* gene in pre-Sertoli cells tips the balance towards increased *SOX9* and *FGF9* expression and the male pathway—genital ridge development proceeds in the direction of testis formation. In the absence of SRY, the pro-female factors dominate allowing for upregulation of β-catenin, favoring the development of the ovary.

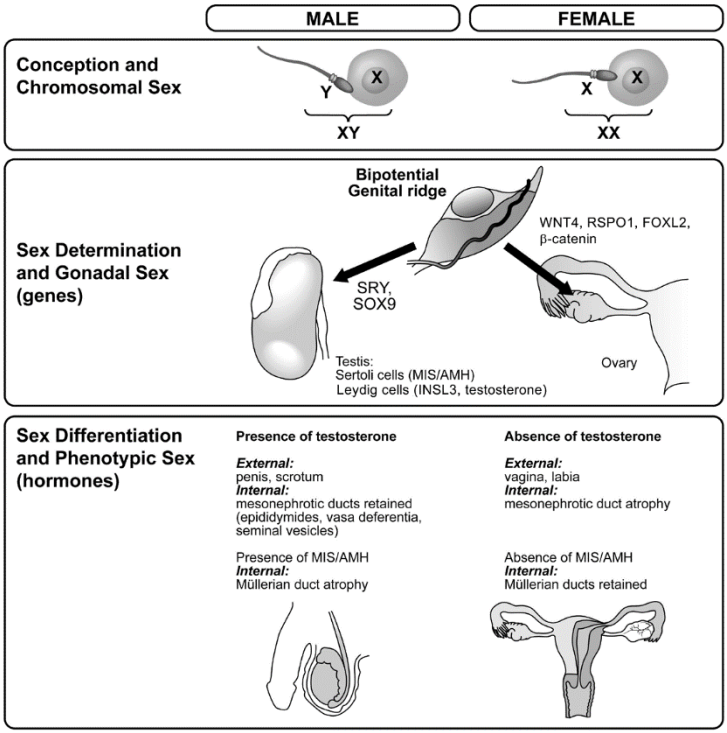

Figure 1. Overview of biological sex in mammals.

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Chapter 27
How do environmental cues affect male reproduction?

William W. Wright

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 season. The timing of when mating occurs ensures that females have a maximal food supply during the 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 specific breeding season restricts the biological and behavioral costs of breeding to the time of year when females are fertile. The onset of the breeding season has specific physiological underpinnings and is associated with activation of the hypothalamic-pituitary-testis axis. Thus, the synchronization of this axis to specific environmental cues is important to the reproduction of males of many species.

While men are not seasonal breeders, there is evidence that season and other environmental cues affect human male reproduction. Herein we summarize data on the effects of season, time of day, temperature, and nutrition on sperm numbers, morphology, or function and on serum levels of testosterone (T) and Luteinizing Hormone (LH).

The effects of season and time of day on male reproduction.

Even though modern societies buffer most of their inhabitants from changes in the seasons, these changes nonetheless affect reproduction. Conceptions vary seasonally in many countries in the Northern hemisphere, with peaks occurring in late spring. However, seasonal effects were substantially greater in the past when communities and homes lacked electric lighting, when homes and workplaces were

not efficiently heated or cooled, and when the ability to obtain, store and distribute nutritious food was limited.

Current data indicate that seasonality in conceptions is associated with changes in numbers of sperm in the ejaculate. Independent studies of healthy men from Boston and from Denmark reported that sperm counts are highest in spring, lowest in summer and intermediate in fall and winter. A study of men from San Antonio, Texas, compared sperm counts in summer and winter. Consistent with the data from Boston and Denmark, Texans had lower sperm counts in the summer.

Numerous studies have tested the hypothesis that a man's serum T levels vary with the season. Results from many but not all studies support this hypothesis, and most of those studies identify peak serum T levels as occurring in late spring. However, others report that T levels peak in late fall or early winter. Discrepancies between different studies may be due to differences in their designs. Some studies have relied on the measurement of testosterone in single serum samples collected from thousands of men at different months and at unrecorded times of day. Differences between individuals and effect of time of day cannot be accounted for during the statistical analysis of those studies

One study of men living in the State of Washington was particularly well-designed. That study used a repeated measures design, where serum hormone levels were measured each month in sixteen healthy men, 19-42 years of age. Importantly each serum sample was collected from a given man at the same time of day. Results demonstrated a significant effect of season on serum T concentrations, with levels of this hormone peaking in May and June.

In many experimental and domestic male animals, serum T levels not only change with season but also with the time-of-day. Diurnal changes in levels of this hormone have also been reported to occur in men, with T levels peaking in early morning and falling to their nadir by early evening.

In summary, even though artificial lighting and central heat and air conditioning buffer most individuals in highly developed countries from seasonal and diurnal cues, there is evidence that these cues still affect a man's reproductive function. Human conceptions, sperm counts and serum testosterone levels peak in the spring. The time of day is also important for serum T levels are highest in early morning.

Physiological mechanisms underpinning seasonal and diurnal changes in a man's serum T levels.

The mechanisms regulating seasonal and diurnal cycles of serum T levels in men are poorly understood. However, data support the conclusion that LH is an important proximal regulator. The previously noted study of men from Washington State demonstrated not only that serum T levels were elevated in May and June, so, too were their serum LH levels. Changes in serum T and LH levels throughout the day are also significantly correlated. Multiple studies of testosterone and LH levels in serum collected every 10-20 minutes from healthy men have revealed a significant relationship between spikes in serum LH levels and spikes in serum T levels; a spike in LH is followed within one to two hours by a spike in testosterone.

What remain to be identified are the molecules and mechanisms regulating diurnal and seasonal changes in serum LH levels of men. In species that are strict seasonal breeders, the pineal gland, and its hormonal product, melatonin, play a significant role in the regulation of LH secretion by gonadotropes. In all seasonal breeders that have been studied, melatonin levels are higher at night than in the day and melatonin secretion decreases as days grow longer and increases as days grow shorter. Depending on whether a species breeds during the summer or winter, a decrease in serum melatonin increases or decreases secretion of LH, respectively. 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 T 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 inhibits the ability of long (summer-like) days to stimulate spermatogenesis in Djungarian hamsters. While mechanisms of action of melatonin are not completely defined, in some species, melatonin increases GnRH secretion. In other species, melatonin suppresses LH secretion, or suppresses gonadotropin-stimulated steroidogenesis. However, a role for melatonin in seasonal changes in human male reproduction has not been established. While numerous human studies have reported that serum melatonin levels peak in the night, changes in day length do not consistently alter this hormone's levels. Furthermore, there is no convincing evidence that a sustained increase in serum melatonin levels affects serum LH or T levels in men. Thus, it remains an open question as to whether melatonin plays an important role in human male reproduction.

The effects of heat on male reproduction.

While there is no definitive evidence that changes in day length drive seasonal changes in a man's serum LH levels or sperm counts, there is evidence that very high summer temperatures can affect sperm. A study of the effects of ambient air temperature on sperm in an ejaculate demonstrated that excessive heat and, interestingly, extreme cold, significantly reduce sperm count, concentration, and motility. Effects of high heat are also observed in men who take sauna baths. A single, 15-20 minutes sauna session (80° to 100°C dry heat) was reported to cause swelling of the plasma membranes of sperm in the ejaculate, and ~~to~~ disorganization of the arrangement of their mitochondria. Repeated sauna sessions (2 sessions per week for 3 months) reduced sperm counts, concentration and progressive motility by 50%. A return to normal did not occur until 3 and 6 months after the last sauna bath. It is worth noting, however, that there are no reports that saunas reduce a man's serum testosterone levels.

Nutrition and male reproduction.

Food, like light and temperature is an important component of our environment. Many individuals in highly developed countries have access to adequate nutrition year-round. But some individuals are malnourished because they live in a food desert or suffer from an eating disorder. Thus, it is noteworthy that there is growing evidence that nutrition has a significant impact on male reproduction, even in apparently healthy men. For example, when non-obese men with low sperm counts ate a well characterized, healthy Nordic diet for one week, there was a significant increase in the numbers of motile sperm in their ejaculates. Greek men who strictly adhered to a Mediterranean diet (fresh vegetables and fruit, fish, and whole grains) are reported to have higher sperm counts and motility than Greek men who infrequently eat such a diet.

Effects of malnourishment on male fertility are evident in obese men, who often do not consume a healthy, balanced diet. In a metanalysis of 30 publications, Campbell and colleagues concluded that obese men are more likely to be infertile, to have higher percentage of morphologically abnormal sperm and to have a reduced rate of live births per IVF cycle. Furthermore, clinical studies demonstrate that obese men tend to have lower sperm counts and serum testosterone levels.

In summary, access and consumption of healthy diet has a significant effect on a man's fertility.

Conclusion

While most males in developed countries are less affected by environmental cues than their ancestors, there is evidence that cues from their environment still affect the number and motility of ejaculated sperm as well as serum LH and testosterone levels. The changes in the seasons, the progression of the day, excessive heat and availability and consumption of nutritious food can all impact a man's reproductive function. It follows that changes in the geographic locations of a man's home, when and where he works, and his access to a balanced, nutritious diet may affect his reproductive potential.

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Chapter 28
What activates puberty in the male? What causes early or delayed puberty?

Mary M. Lee

Puberty is the developmental stage denoted by the maturational transition from a juvenile prepubertal state to attainment of sexual and reproductive maturity. In boys, activation of the hypothalamic-pituitary-gonadal axis initiates puberty and first manifests as testicular enlargement. During male puberty, secondary sexual maturation is characterized by growth and virilization of the external genitalia (increase in testicular size, phallic and scrotal enlargement), and the development of pubic, axillary and facial hair and adult apocrine odor. Somatic changes include the pubertal growth spurt, changes in body composition and proportions, increased muscle development and strength, deepening of the voice, and neuroendocrine maturation associated with changes in behavior, mood, and cognition.

Pubertal development: adrenarche and gonadarche

Two physiologic processes underlie the hormonal and physical changes associated with puberty. The first is adrenarche or maturation of the adrenal cortex marked by a shift in the pattern of adrenal steroidogenesis. The maturation of the zona reticularis is ACTH independent and results in increased secretion of dehydroepiandrosterone sulfate and other adrenal androgens. The adrenal androgens stimulate growth of pubic and axillary hair, apocrine odor, and acne (termed adrenarche or pubarche). The timing of adrenarche is influenced by familial and ethnic factors as well as adiposity and prematurity. The genetic regulation of adrenarche is not fully delineated but it typically precedes activation of the hypothalamic-pituitary gonadal (HPG) axis and gonadarche, the second pubertal process.

An increase in the amplitude and frequency of secretion of GnRH pulses is necessary to stimulate pulsatile release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the

pituitary gland. The secretion of LH and FSH drives maturation of the testes and increases secretion of sex steroids (Chapter 2). Pituitary FSH stimulates proliferation and differentiation of the Sertoli cells to form the seminiferous tubules to support spermatogenesis, while LH is the main stimulus for increased secretion of the virilizing hormones, testosterone and its more active metabolite, dihydrotestosterone. FSH acts in concert with androgens to stimulate germ cell proliferation and maintain spermatogenesis.

A shift in the balance of the network of central neuro-inhibitory modulators and stimulatory neuromodulators occurs at the onset of puberty. This transition from the juvenile to the pubertal state is marked by a decrease in the tonic inhibition of the GnRH neurons by a group of genes identified to inhibit the HPG axis, such as neuropeptide Y, GABA, Dyn, MKRN3, and ghrelin as well as by increased secretion of kisspeptin and other HPG excitatory genes. Several genes have been identified to be critical to the initiation of puberty, such as glutamine, KISS1 (kisspeptin) and TAC3 (neurokinin B, NKB) and their respective receptors. Kisspeptin, a potent stimulator of GnRH release, signals through a G protein coupled receptor 54 (GPR54 or KISS1R), expressed by GnRH neurons. KISS1 expression is low during childhood and increases at puberty. In addition to genetic influences on pubertal timing, pubertal onset is also modified by other factors such as nutritional status, chronic illness, strenuous physical activity, environmental stress, and mental health. These factors may affect the onset and tempo of puberty via epigenetic mechanisms and impede the attainment of full reproductive capacity. Mutations in many of these inhibitory and stimulatory genes have been linked to disorders of pubertal development.

During embryonic development, the initial activation of the hypothalamic neuronal network results in pulsatile GnRH secretion and stimulates fetal testicular Leydig cell production of testosterone to promote internal and external genital masculinization. The fetal Leydig cells continue to secrete testosterone after birth for a few months to stimulate ongoing differentiation of the external genitalia. During this “mini-puberty” of infancy, despite the high levels of gonadotropins and androgens, the seminiferous tubule of the infant does not undergo further maturation because androgen and FSH receptor signal transduction pathways in the infant Sertoli cells remain immature. In later infancy, the GnRH-gonadal axis becomes inhibited and remains quiescent during childhood.

The 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 by late infancy, leads to inhibition of GnRH release during prepubertal development, and the second transition occurs when there is reactivation of intermittent GnRH release at the onset of puberty. The term GnRH pulse generator was coined to describe the main "trigger" for the onset of puberty and has been used to describe the role of kisspeptin in regulating pubertal onset, although it is now appreciated that there is a complex interplay of inhibitory and stimulatory genes responsible for activation of the HPG axis at puberty.

Assessing pubertal development

Pubertal development is assessed using a genital staging scale first developed by Dr. James Tanner that evaluates adrenarche and gonadarche independently based upon visual inspection. The scale is scored from 1 (prepubertal) to 5 (sexual maturity) for genital development (G1-5) and pubic hair growth (P1-5). Measurement of testicular volume yields a more accurate assessment of male pubertal development. An orchidometer or Prader beads are a string of elliptical beads ranging in size from 1 cc to 25 or 30 cc. Prepubertal testes are 1-2 cc in size; a 3 cc testis is indicative of pubertal onset, and full maturity is seen with testes of 20-30 cc in volume. Typically, development of adrenarche and gonarche are synchronized and seldom discordant by more than two stages. The average onset of puberty in boys is now 10-11 years with a normal range from 9-13 years. Progression through puberty from stage 2 to 5 occurs over approximately 3.5 to 5 years. In boys the pubertal growth spurt occurs during mid-to late puberty, and sexual maturity is attained by age 15-16 years on average. Over the past 50 years, secular shifts in the age of onset of puberty have been reported world-wide. This has been attributed to factors such as improved nutrition as well as exposures to environmental endocrine disrupting chemicals.

Precocious puberty: causes and treatments

Any deviation from the average (+/- 2 SD) age of puberty that is inconsistent with familial patterns might be considered a disorder of pubertal development. Early puberty is less common in boys than in

girls and warrants close examination to determine if puberty is isolated to adrenarche (only pubic hair with or without other findings of virilization) in conjunction with prepubertal testes or if the gonads are also enlarging and if there are other somatic signs of excess androgen exposure. Potential consequences include tall stature during childhood, sexual maturity and appearance discordant with chronologic age and with emotional/social maturity, and advanced skeletal maturation leading to early epiphyseal fusion and cessation of linear growth resulting in adult short stature. Adrenarche and virilization without growth in testicular size suggests either exposure to exogenous androgens or secretion of androgens from congenital adrenal hyperplasia or a virilizing tumor. Congenital adrenal hyperplasia represents a group of disorders with 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 shunting to the pathway of adrenal androgen secretion. True precocious puberty may be idiopathic but organic causes must be excluded. The first step is to differentiate between a central versus peripheral process. Central precocious puberty with premature activation of pulsatile GnRH secretion can be caused by a CNS process--brain tumor or hypothalamic activation due to a central inflammatory disorder, brain trauma, infection, or radiation. A condition mimicking central precocious puberty can be caused by tumors located in the liver, mediastinum, or central nervous system that secrete human chorionic gonadotropin which binds to testicular LH receptors and stimulates testosterone secretion. Central precocious puberty can be suppressed using long-acting GnRH receptor analogues such as leuprolide acetate and histrelin. These GnRH receptor agonists lack the pulsatile pattern that is needed to stimulate pituitary gonadotropin secretion and instead the high levels lead to a negative feedback mechanism to inhibit FSH and LH secretion.

Precocious puberty may also result from GnRH independent androgen secretion caused by familial autosomal dominant male limited precocious puberty (LH receptor activating mutations/ testotoxicosis), McCune-Albright syndrome (GNAS somatic mutation), or Leydig cell tumors. 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 as central puberty can ensue.

Delayed puberty: causes and treatments

Delayed puberty is more common than precocious puberty in boys. The most common cause is a familial variant of normal, termed constitutional delay of growth and development (CDGD), i.e., "late bloomers". Often, boys with CDGD are referred to the pediatric endocrinologist because they appear noticeably younger and shorter than their peers and are less muscular with no signs of puberty. For some boys, reassurance is sufficient, particularly if there is a strong family history; for others, the delayed puberty and growth may be associated with depression, anxiety, and school failure that may lead to major psychosocial dysfunction. As genes are identified that mediate the tempo of puberty, mutations have been identified that are responsible for familial delayed puberty. A short course of androgen therapy can be beneficial in accelerating linear growth and pubertal development and alleviating the psychosocial concerns.

Organic etiologies of delayed or absent puberty should be differentiated as centrally or peripherally mediated. Primary gonadal failure is typically accompanied by elevated gonadotropins and can be due to testicular trauma, gonadotoxic drugs, radiation, intrauterine torsion, or genetic causes. The most common genetic condition is Klinefelter syndrome (Chapter 37) (47,XXY) which can cause a late onset of puberty or incomplete progression through puberty. Single gene disorders, gonadal dysgenesis, and XX sex reversals are other rarer causes. Central causes of delayed puberty may include a mutation in one of the myriad of genes involved in the control of GnRH or gonadotropin secretion or action, chronic illnesses, psychosocial factors such as depression or stress, and impaired nutrition.

Conclusion

The detailed physical examination, growth pattern, and family history can provide valuable diagnostic clues for syndromic or associated findings. Skeletal bone age film is useful for assessment of both early and late puberty. A bone age that is discordant with chronological age reflects the key contribution of sex steroids to skeletal maturation and linear growth. Laboratory evaluation for delayed puberty includes serum LH, FSH, and testosterone measurement. While testosterone concentrations are subnormal in both central and primary causes of delayed puberty, 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. Treatment of the primary illness and improvement in mental health or nutritional status can often help in hypothalamic hypogonadism.

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Chapter 29
How do paternal factors such as diet, smoking, stress, and environmental chemical exposures affect germ cell mutations?

Jonatan Axelsson, Habiballah Shojaeisaadi, Francesco Marchetti and Carole Yauk

What are mutations and why do they matter?

Mutations are changes in the DNA sequence affecting one or several complementary base-pairs. Point mutations, or single nucleotide variants (SNV), are changes in a single base, whereas insertions or deletions of base-pairs (INDELS) can range in size from a single base-pair to tens of thousands of base-pairs.

In general, most mutations are silent (i.e., have no observable effect on the organism's phenotype), but they can also cause devastating genetic disorders or even, in very rare instances, lead to a beneficial adaptation. A mutation in protein-coding genes or their regulatory regions can alter the amino acid sequence of the protein or gene expression levels resulting in a manifested genetic disease.

Mutations that occur in somatic cells can cause diseases such as cancer. However, mutations can also occur in germ cells that serve as the progenitors for the offspring. These inherited germline mutations will be present in the entire organism and have a higher chance to result in a variety of adverse phenotypes. Therefore, the faithful transmission of DNA through the germ cells from parents to offspring is critical to individual and population health. Thus, highly accurate DNA repair mechanisms have evolved to protect the integrity of the genome and prevent mutations. Indeed, germ cell mutation frequencies are approximately one order of magnitude lower than somatic mutation frequencies.

How do paternal factors affect germ cell mutations?

What are the consequences of germ cell mutations?

Germ cell mutations can have pre- and postnatal effects on the embryo or child, respectively. Mutations affecting prenatal development can lead to pregnancy loss, whereas mutations compatible with life can be associated with postnatal effects including malformations, cancer predisposition syndromes and neurodevelopmental disorders such as autism, epilepsy and intellectual disability. Moreover, inherited germline mutations can be transmitted from one generation to the next and spread through families and populations.

What is the difference between *de novo* mutation and germline mosaicism?

A mutation in an individual that is not found in the parents is known as a *de novo* mutation. *De novo* mutations are a major cause of severe diseases that can arise in early childhood or later in life and account for almost 30% of rare diseases. The rate of *de novo* SNV mutations in humans is about 1.2×10^{-8} mutations per nucleotide per generation (which corresponds to approximately 60-80 mutations per genome). Around 80% of *de novo* mutations are of paternal origin and may result from (a) a mutation in the germ cell itself, or (b) DNA damage in the male germ cell that was not repaired correctly in the fertilized egg. However, mutations may also appear during early embryonic cell divisions, leading to a mixture of cells in the fetus with and without the mutation – a state called mosaicism. These mosaic mutations can be distributed to both somatic and germ cells of the developing organism, or only affect a specific population of cells.

What are the general causes of germ cell mutations?

Mutations may occur spontaneously but can also be induced by exogenous factors that damage the germ cell DNA. Damage introduced during DNA replication can be converted into mutations if not properly repaired. Thus, mutations primarily arise during phases of spermatogenesis with active replication and cell division (Fig. 1). There is evidence to suggest that germ cell susceptibility to mutation may vary across life stages but more research is needed in this area.

Similarly, chromosomal damage can occur during mitosis and meiosis in germ cells (Fig. 1). DNA recombination can be a source of

mutations and abnormalities in chromosome number or structure, with important reproductive and clinical consequences. Finally, DNA damage can accumulate in post-meiotic phases of spermatogenesis when DNA repair mechanisms decline; this DNA damage can be converted into mutations after fertilization.

A large number of exogenous agents (*e.g.*, ionizing radiation and many chemicals) are known to induce mutations in animal germ cells by various mechanisms. These agents are collectively called mutagens. Mutagens can occur naturally or be man-made and include acrylamide, benzo(a)pyrene (a combustion by-product), and chemotherapeutic agents, among others. For example, both tobacco smoke and air pollution cause mutations in mouse sperm and paternal exposure to mutagenic compounds present in tobacco smoke cause genome-wide *de novo* mutations in the offspring of the exposed male mice. These studies demonstrate a dose-dependent association between mutagenic exposure and mutations in both sperm and offspring.

What are the known and suspected causes of germ cell mutations in humans?

Paternal age is strongly associated with the number *de novo* mutations in human offspring. On average, ~1.5 additional *de novo* mutations are transmitted to offspring with every year that a man delays fatherhood. Interestingly, this paternal age effect varies between different families, suggesting that environmental factors (such as diet, smoking, stress, and environmental exposures) may play a role. In line with this, a reduced number of *de novo* mutations has been reported in an Amish population, who have different lifestyles and exposures than people living in urban environments.

Despite the strong evidence in animals, there is still no consensus on whether human germ cell mutagens exist. The strongest evidence for an association between an exposure and germ cell mutations in humans at present is smoking. Tobacco smoke contains many established mutagens that cause DNA adducts, strand breaks and oxidative damage, and is the largest cause of cancer in the world (with cancer being largely dependent on mutagenesis). It is well established that the sperm of smokers have elevated levels of DNA damage. Paternal smoking is also associated with malformations and childhood cancer in offspring. However, although the children of smoking fathers appear to have

How do paternal factors affect germ cell mutations?

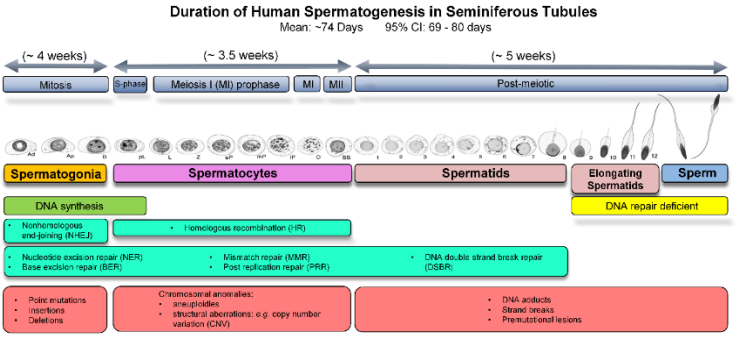

Figure 1: Schematic diagram of the developmental sequence of germ cell types present in the seminiferous tubules during human spermatogenesis. The approximate periods of DNA synthesis, active DNA repair (with specific types of repair indicated), inactive DNA repair, as well as the most common types of genetic damage that are induced in each germ cell type are shown. Ad, A-dark spermatogonium; Ap, A-pale spermatogonium; B, B spermatogonium; pL, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; eP, early pachytene spermatocytes; mP, mid-pachytene spermatocytes; lP, late pachytene spermatocytes; D, diplotene spermatocytes; SS, secondary spermatocytes; 1–12, first 12 steps in the development of spermatids. The drawings of the germ cell types are taken from *Biology of Reproduction (2013) 89(3):60, 1–10.*

an elevated number of mutations in a region of non-coding repetitive DNA, a significant increase in other types of mutations such as SNVs in the children of smoking fathers has yet to be reported. Additional potential germ cell mutagens in humans are ionizing radiation, chemotherapeutic drugs, dioxin, and air pollution.

Exposures that have been associated with numerical and structural chromosome abnormalities in human spermatozoa include smoking, alcohol consumption and occupational exposure to benzene. Air pollution and ionizing radiation are also associated with sperm DNA fragmentation.

Studies in humans on the influence of diet on germ cell mutagenesis are limited. However, the vitamin folate (for which preconception supplementation is arguably the most effective intervention against human congenital disorders) appears to protect against aneuploidy in human sperm. At present, studies on

the impacts of social, psychological, and physiological stresses on human germ cell mutations are lacking.

What role do mutations play in male reproductive function?

As much as 20% of unexplained male infertility is associated with *de novo* mutations in genes. This may be related to the higher rate of structural mutations in the Y chromosome (in which microdeletions can cause infertility), as compared to the overall rate of point mutations in the genome. *De novo* structural mutations, including copy number variants, affect about 100 times more nucleotides per generation than single base substitutions and, as with SNVs, most often arise in the paternal gamete.

Certain mutations in germ cells that show a paternal age effect, such as the gene responsible for achondroplasia, a common form of dwarfism, appear to give an advantage to the germ cell clone (the selfish spermatogonial selection hypothesis) despite causing severe disease in the offspring. These findings support the UK's guideline recommending an upper limit of < 46 years old age for sperm donors. Taken together, the data suggest that, as with women, men also have a 'biological clock'.

Couples who are carriers of disease-causing genes can undergo *in vitro* fertilization (IVF) and preimplantation genetic testing (PGT) to achieve a healthy pregnancy. During this process, embryos are screened for the particular mutation, after which embryos without the targeted mutation can be transferred to the mother. However, genetic screening for *de novo* mutations, which could occur anywhere in the genome, is not yet being applied in PGT. Advances in assisted reproductive techniques (ART) are also allowing men with poor semen quality to become fathers. A recent study reported genome-wide *de novo* SNV mutations (most of which were paternal) to be higher in children conceived after ART than in children from natural conceptions. This is an area that merits additional investigation.

Conclusions

Animal studies have repeatedly shown that *de novo* mutations in offspring can be caused by a wide range of paternal exposures including tobacco smoke and air pollution. It seems hard to believe that no exposure would be able to cause such mutations in humans. Despite the lack of consensus within the expert community, the

weight of evidence supporting the existence of human germ cell mutagens continues to grow. Exposures to ionizing radiation, several chemotherapeutic agents, and tobacco smoking have been associated with genetic changes in human sperm. What remains to be established is a significant increase in *de novo* mutations in the children of exposed fathers. In this context, a recent study found a few cases of increased *de novo* SNV mutations in children of men who had been treated with chemotherapeutic agents. In addition, it is now firmly established that paternal age at the time of conception is strongly and positively correlated with the number of *de novo* mutations occurring in their children. A major impediment to the study of human germ cell mutagenesis has been the lack of sensitive and accurate methodologies to quantify *de novo* mutations. Emerging genomic technologies, including highly accurate next-generation sequencing approaches, are anticipated to address this methodological gap in the future and provide proof of the existence of human germ cell mutagens.

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Chapter 30
What male contraceptives currently exist and what is the outlook for male hormonal contraception?

John K. Amory and Stephanie T. Page

Current male contraception: Vasectomy and condoms

There currently exist two efficacious, approved contraceptive options for men: vasectomy and condoms. Together, condoms and vasectomy account for 20-30% of contraception in the US. In other countries, the use of condoms and vasectomy vary significant based on local availability and cultural acceptability.

Vasectomy is a safe outpatient surgery usually performed under local anesthesia. During a vasectomy, the vas deferens is severed and the ends ligated or cauterized through a small scrotal incision. Vasectomies are a highly effective method of permanent contraception with a failure rate of less than 1%. Most failures occur early after the procedure and before all the sperm have been cleared from the proximal vas, although late failures due to vas re-canalization have been reported. Most surgeons currently utilize the "no scalpel" technique pioneered in China, in which a puncture is made midline in the scrotal raphe with scissors. Drawbacks to vasectomy include a 3-4 month delay in the onset of azoospermia, post-vasectomy pain (<5%) and rare infections. While most post-vasectomy pain resolves quickly, a small number of men will experience chronic scrotal discomfort requiring reversal. Vasectomy is most appropriate for men who no longer wish to father children, since surgical reversal does not always restore fertility and is costly.

Condoms made of animal intestine have been used as a means of male fertility control for at least several hundred years. Since around 1920, most condoms have been made of latex rubber, have improved reliability and are less expensive than other options. The use of condoms during sex also affords some protection against most sexually transmitted diseases including HIV/AIDS. Unfortunately,

condoms have a middling contraceptive efficacy long term, with pregnancy rates of 10-15% per year in couples using condoms as a sole method of contraception; this contrasts with female contraceptive methods such as intrauterine devices and oral pills which have failure rates of 1 and 9% respectively with typical use. Condom failure is frequently due to inconsistent use or breakage, although pregnancy can occur even with correct and consistent use. In addition, latex allergies can be a problem for some users. For these men, polyurethane condoms are a reasonable alternative.

Experimental hormonal male contraceptives

Efforts are ongoing to develop new methods of contraception for men. The approach that has been tested most extensively are hormonal male contraceptives. Hormonal male contraceptives rely on the administration of testosterone, which functions as a contraceptive by suppressing the secretion of luteinizing hormone (LH) and follicle-stimulating (FSH) hormone from the pituitary (Fig. 1). The absence of normal circulating concentrations of LH and FSH deprives the Leydig cell of the signal necessary for steroidogenesis leading to markedly reduced concentrations of intratesticular testosterone. The low intratesticular testosterone coupled with the low FSH deprives the seminiferous tubules of the signals needed to promote spermatogenesis and results in effective contraception in a majority of men. Male hormonal contraception is well tolerated, fully reversible, and appears be associated with a low rate of serious adverse effects. Unfortunately, regimens in which testosterone is used alone fail to completely suppress spermatogenesis in a subset of men. Therefore, combinations of testosterone and progestins, which synergistically suppress gonadotropins, are currently employed in most studies.

In most fertile men, sperm concentrations exceed 15 million/ml (normal range 15-200 million/ml). The absence of detectable sperm in the ejaculate, a condition called azoospermia, makes fertilization impossible. In prior male contraceptive studies, a sperm concentration suppressed to below 1 million/ml, “severe oligospermia,” is associated with a pregnancy risk of approximately 1% per year. Therefore, achieving severe oligospermia in all men is considered a reasonable near-term goal of male contraceptive development. In addition, because male hormonal contraceptives inhibit sperm production, it takes 3-4 months of treatment until the sperm concentration in most men is suppressed to under 1 million/ml.

Several large male contraceptive efficacy studies have been performed using hormonal contraceptives. The first two were conducted by the World Health Organization conducted two large, multicenter trials of weekly injections of testosterone enanthate for male and enrolled over 600 couples. The first demonstrated the high efficacy of hormonally-induced azoospermia. The second also allowed men with either azoospermia or oligozoospermia to rely on the regimen of testosterone injections for contraception. In this second study, there were no pregnancies fathered by the men who

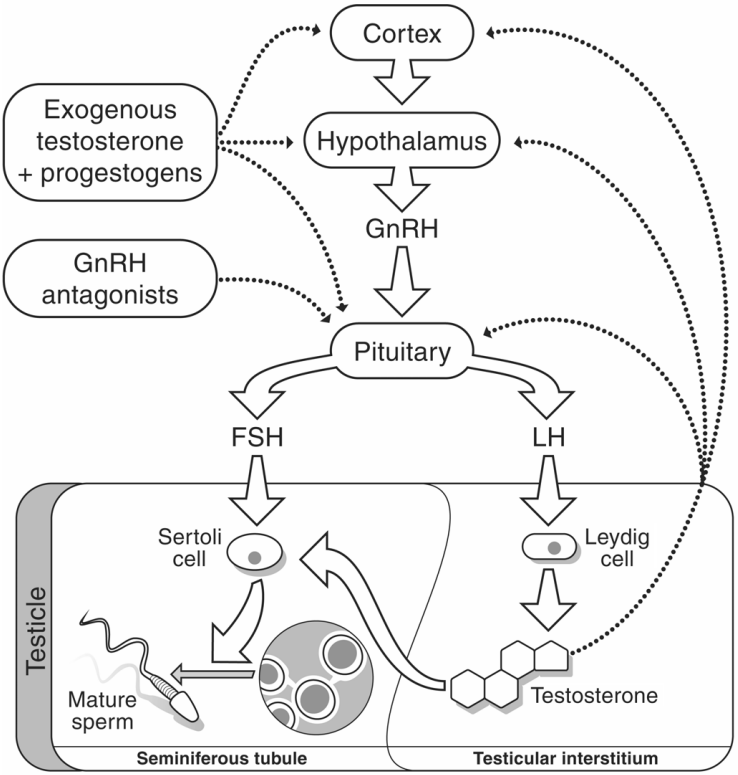

Figure 1. Spermatogenesis and male hormonal contraception. Solid arrows, promotes spermatogenesis; dashed arrows, inhibits spermatogenesis. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone. Negative feedback of testosterone occurs at the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

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 for an overall failure rate of 3.4%. Recovery to normal sperm concentrations occurred in all participants after the testosterone injections were discontinued. These two studies demonstrated that testosterone injections are a safe, reversible and highly effective contraceptive in a majority of men. However, they also demonstrate that pregnancy remains possible even at very low sperm concentrations and, for unknown reasons, a small group of men continue to exhibit low level spermatogenesis despite hormonally-mediated gonadotropin suppression.

Side effects in these trials included a 10-20% decrease in serum HDL- cholesterol, occasional acne attributable to the supraphysiologic testosterone dosing, 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.

Since the WHO trials research in hormonal male contraception has focused on formulations of testosterone that can be administered less frequently, such as testosterone undecanoate and combinations of testosterone and progestins to maximize gonadotropin suppression. Monthly injections of testosterone undecanoate have been studied in over 1000 Chinese men with a contraceptive efficacy of 95%. Injections of a combination of testosterone undecanoate and noresthisterone acetate were studied in a third WHO sponsored study and appeared promising in terms of contraceptive, but this study was ended early due to mood disorders in some men. Currently, the NIH is sponsoring a large, multinational contraceptive efficacy trial employing a combination of testosterone and nestorone topical gels, which had shown excellent suppression of spermatogenesis in earlier studies. Data from this study should be available in 2023-24. Lastly, the NIH has two promising novel steroids, dimethandrolone and 11-beta-methyl nortestosterone, that have inherent androgenic and progestational activity and are orally bioavailable that seem promising in early clinical studies as a single agent “male pill”.

Underlying this work is a mystery. Why do a small minority of men fail to fully suppress their spermatogenesis despite profound suppression of gonadotropins by male hormonal contraceptives?

Since there are no 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. Further studies of the control of spermatogenesis are needed to optimize this approach to male contraception.

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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Chapter 31
What are the current approaches to non-hormonal male contraception?

John K. Amory

Experimental non-hormonal male contraceptive approaches do not rely on the administration of hormones or compounds that block hormone secretion. Non-hormonal male contraceptives may have some advantages compared to hormonal male contraceptives as they avoid any impact on testosterone concentrations and therefore would be less likely to alter sexual function, sex drive or body composition and would not prevent participation in high-level sporting events that exclude the use of exogenous androgens. In addition, oral delivery may be more feasible with non-hormonal compared to hormonal male contraceptives given the difficulty with oral dosing of testosterone. The following sections highlight past and ongoing efforts to develop non-hormonal contraceptives for men.

Gossypol

The first extensively studied non-hormonal male contraception was gossypol. Gossypol is a large molecule purified from the seeds of a cotton plant grown in China. Gossypol was tested as a non-hormonal male contraceptive in clinical trials enrolling more than 8800 men in China in the 1970s and 1980s. In these studies, gossypol administration reduced both sperm production and sperm motility via an unknown mechanism. Most treated men developed azoospermia and Gossypol had a greater than 90% efficacy in pregnancy prevention. Unfortunately, side effects including hypokalemia and hypokalemic periodic paralysis occurred in about 1% of treated men. In addition, spermatogenesis did not fully recover in 10-20% of men. Despite significant efforts to chemically modify the structure of gossypol to reduce the risk of side effects, the study of Gossypol for non-hormonal male contraception has been largely abandoned.

Triptolide/Triptonide

A second naturally-derived male contraceptive compound studied in China was the herb *Trypterigium wilfordii*, the active compound of which is called triptolide. *Trypterigium* had been in traditional Chinese medicine for many centuries for the treatment of arthritis. Clinical study of patients treated with this compound showed that *Trypterigium* administration impaired sperm motility and decreased sperm counts. Unfortunately, as was the case with gossypol, several men experienced irreversible suppression of spermatogenesis and other side effects, causing the abandonment of work studying this compound as a reversible male contraceptive. Recently, a related compound, triptonide, has shown potent contraceptive properties in animal studies without the toxicities observed with triptolide. Further work with this compound will be of great interest.

Adjudin

The compound Adjudin was studied as a non-hormonal male contraceptive in animal studies in the early 2000s. The administration of Adjudin to rodents interferes with the ability of spermatids to adhere to Sertoli cells. Because of this, the spermatids undergo premature spermiation resulting in the production of non-functional spermatozoa that are incapable of fertilization. In rats, administration of 50 mg/kg of adjudin twice weekly induced 100% infertility after 5 weeks of treatment without changing serum gonadotropins or testosterone concentrations. Unfortunately, several animals experienced liver inflammation in a 29-day study at this dose. Follow-up work conjugated Adjudin to a FSHβ mutant, specifically targeting it to Sertoli cells, thereby reducing systemic exposure. Unfortunately, this approach proved prohibitively costly, and further study in either animals or humans of this conjugate was not undertaken.

EPPIN

EPPIN is a protein located on the surface of the sperm. EPPIN functions in liquefaction of the ejaculate; the absence of liquefaction severely impairs sperm motility. Initial immunization studies in male non-human primates demonstrated that a majority could be immunized against EPPIN. Notably, these males were mated and were unable to father pregnancies. Importantly, the animals re-gained fertility after

cessation of the injections. After this proof-of-principle immunization study, this research group has focused their work on developing small molecules that inhibit EPPIN binding to the protein semenogelin. A recent publication demonstrated that IV administration of the small molecule EP055, temporarily reduced sperm motility by 80% in male macaques. The group is now working on the development of potent, oral compounds in animal studies. Hopefully, continued work on this approach will result in a pill that can effectively reduce sperm motility for testing as a male contraceptive.

BRDT Inhibition

The bromodomain protein, BRDT, is required for meiosis. Intriguingly, men with mutations in the *Brdt* gene have infertility and semen analysis reveal abnormal sperm heads and poor motility. In 2012, a group showed that JQ1, a small molecule that potentially inhibited BRDT function, was shown to reversibly suppress fertility in a murine model. Unfortunately, JQ1 inhibits other bromodomain proteins, leading to toxicity. This group is performing structure-activity modeling of JQ1 in efforts to develop a BRDT specific inhibitor, retaining the contraceptive action while minimizing the potential for side effects.

Retinoic Acid Receptor Antagonists

It has been known since 1925 that vitamin-A (retinol) is essential for sperm production and male fertility. All of the effects of retinol appear to be mediated by retinoic acid. Retinoic acid functions via binding to a family of retinoic acid receptors (RARs), which serve to regulate gene expression. Gene knockout experiments have shown that mice with deletion of one of several of the RAR are sterile. Based on these observations, several groups are working on developing non-hormonal approaches to male contraception based on the blockade of retinoic acid function or biosynthesis.

One example of such a compound is BMS-189453, which was described in the early 2000s. This compound is an oral RAR pan-antagonist. Initial one-month studies of BMS-189453 at doses of 15, 60, or 240 mg/kg to rats lead to marked testicular degeneration and infertility, but also liver toxicity. A second group of investigators followed-up on these earlier studies, testing lower doses of BMS-18945, demonstrating efficacy at sperm suppression without the liver toxicity observed with larger doses. For example, mice treated

with 2.5-5 mg/kg for 4 weeks were completely sterile by 4 weeks of treatment with return to fertility 12 weeks after the cessation of treatment. A specific retinoic acid-alpha antagonist, YCT 529, appears to be effective as a contraceptive in rodents and larger species and may enter clinical testing soon.

Retinoic Acid Biosynthesis Inhibitors

Almost sixty years ago, the administration of WIN 18,446 was shown to dramatically suppress sperm production in men and was studied as the first non-hormonal male contraceptive in almost 100 men. Unfortunately, it was discovered that men taking WIN 18,446 had serious "disulfiram reactions" characterized by vomiting, sweating and palpitations when they drank alcohol while taking WIN 18,446. Because of these severe disulfiram reactions, further study of WIN 18,446 as a male contraceptive stopped. In 2011, it was shown that WIN 18,446 functioned via inhibition of testicular retinoic acid biosynthesis. It was further demonstrated that WIN 18,446 inhibited two enzymes called aldehyde dehydrogenase ALDH1A1 and ALDH1A2 that are the final step in retinoic acid production. Work in this area is now focused on the production of novel that specifically inhibit ALDH1A1 and 1A2 without causing disulfiram reactions, which are mediated by a similar enzyme ALDH2.

Gendarussa

An Indonesian traditional medicine called *Justicia gendarussa* has been reported to be used as traditional form of contraception by men in Papua New Guinea. The active ingredient is thought to be a flavonoid called gendarusin A. Some data on contraceptive efficacy for this compound has been reported in abstract form, but not published. In addition, the mechanism of action remains unclear. Therefore, additional information will be needed to determine whether this is a viable approach to developing a non-hormonal male contraceptive.

Vas Occlusion Methods

Several research groups have conducted research directed towards developing methods to reversibly plug the vas deferens since the 1970s. Reversible vas occlusion is an attractive approach to male contraception, as the initial vasal obstruction could provide long-

lasting contraception. Ideally, the man could later have the obstruction removed, and have his fertility restored. An Indian vas occlusion device called RISUG (reversible inhibition of sperm under guidance) has been studied in several clinical trials in men. The initial procedure is performed under ultrasound guidance. Specifically, a solution of styrene maleic anhydrate is injected into the vas deferens bilaterally, effectively occluding the vas and preventing the passage of sperm during ejaculation. Data from several small clinical trials of RISUG is available. Taken together, these studies demonstrate effective contraception over periods of up to one year in men. Unfortunately, no data from large-scale trials or demonstration of reversibility have been published.

A re-formulation of RISUG, called "Valsalgel™" in the US was tested as a contraceptive for one year in rabbits, and monkeys. After reversal, however, the sperm of the rabbits no longer had acrosomes, possibly due to inflammation in the vas. Therefore, as was the case with in the Indian studies, it remains unclear if RISUG is truly reversible. In similar work, Vas occlusion devices using medical-grade silicone and polyurethane were studied in China in the 1990s. However, these devices also experienced incomplete recovery of sperm parameters after attempted reversal. Newer work from China and the US with intravasal hydrogels, which may be more reversible, is showing promise and may be entering clinical trials soon.

Thermal Contraceptives

The application of heat to the testes can impair spermatogenesis and has been studied as a contraceptive in small studies. In one study of five men, the long-term use of specialized underwear that elevates the testes to near the outer inguinal ring lead to reversible suppression of sperm production and abnormal sperm. Additional testing of this approach with larger groups is warranted.

Conclusions

Contraception provision is essential for the prevention of unintended pregnancy. Given the limitations of currently available methods of contraception, there is a great deal of interest in the development of novel male contraceptive methods. Several non-hormonal approaches have been studied in mostly pre-clinical studies, but large-scale human studies to determine their safety and

efficacy are lacking. Additional research is required to meet the unmet need of male-driven methods of birth control.

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Chapter 32
How is male infertility defined? How is it diagnosed?
Epidemiology, causes, work-up

Peter N. Dietrich and Jay I. Sandlow

An estimated 1 in 7 couples experience difficulties with conceiving. While societal and cultural biases tend to focus on the female side of fertility, male factor is present in up to 50% of infertile couples. Generally, the diagnosis and workup of male infertility is prompted by abnormal semen parameters or inability to transport semen, although novel research has brought to light markers of sperm quality which also contribute to fertility. Any pathology which prevents transport or production of sperm can be attributed as male infertility. The term "subfertility" has also been used to describe this and can be used interchangeably with infertility. A thorough evaluation of the male partner, along with concurrent female evaluation, is essential in infertile couples to aid in counseling and treatment. Male infertility has also been shown to be a harbinger for future diseases, hospitalization, and early mortality (Chapter 64). The male partner workup provides a unique opportunity for the andrologist to perform an early overall health evaluation in a young population unlikely to have regular medical visits.

The causes of male factor infertility are varied, ranging from issues of sperm quality and/or production to abnormal transport (emission, ejaculation, or obstruction). Primary infertility is defined as the inability to conceive with any partner, while secondary infertility is defined as prior ability to conceive but subsequent fertility difficulties. Varicoceles are the most common cause of male infertility, being present in 40% of men with primary infertility and over 80% of men with secondary infertility. Approximately 25% of patients will have idiopathic or unexplained infertility, although recent guidelines reclassify many of these patients as having hypogonadism, which was previously present in 9% of patients. Other causes include primary testicular failure (9%), obstruction (6%), and cryptorchidism (5%). Anejaculation and retrograde ejaculation make up approximately 2% of male infertility.

The basic evaluation of a male presenting with infertility begins with a thorough history. Male infertility has been associated with higher overall mortality, and thus patients should have a detailed history to assess for concomitant diseases. Duration of infertility, past fecundity, and any infertility treatments should be noted. Past and current medical problems such as genitourinary infections, malignancies, medications and congenital or developmental issues should be evaluated. Special attention is given to past surgeries including genitourinary instrumentation, hernia repairs, orchidopexies, and scrotal surgeries, which may increase the risk of infertility. Female partner age, past fertility, cycle information, and fertility workup are also important factors. Sexual function, change in erections or ejaculation, and timing and frequency of intercourse should be discussed. Family history should focus on infertility, endocrine disorders, malignancy, and cystic fibrosis. The patient's social history can elucidate any modifiable substance use or exposures. Other patient specific information, such as exogenous testosterone use, past trauma, or prior infertility workup can be useful and should be considered in all patients.

A physical exam should be performed both standing and lying down, with focus on general examination of body habitus and development of secondary sex characteristics. Additional characteristics associated with infertility include anosmia (Kallman syndrome) and situs inversus (Kartagener's syndrome). Abdominal exam should focus on the presence of any healed incisions, as patients may have had surgery as a child that they are unaware of. The phallus should be examined for hypospadias, lesions, or other urethral defects. Careful palpation of the testes, vas deferens, epididymides and cords should be performed. Testicular size can be estimated with the use of an orchidometer. Testis size and texture can offer insight into pathology, with small and soft testicles being associated with hypogonadism. Small (3-6cc), firm testicles are associated with Klinefelter's disease, the most common genetic cause of male infertility. Congenital bilateral absence of the vas deferens should prompt cystic fibrosis transport receptor (CFTR) testing in both the patient and the female partner. Unilateral absence should be followed by a renal ultrasound, as wolffian duct development alterations can lead to renal agenesis. Although virtual consultations have become more popular in recent years, it is still recommend that an in-person visit be done for all new infertility patients as a physical exam is invaluable to establishing a diagnosis. At least two semen analyses should be performed, preferably with at least 3 weeks between samples. In

patients with less than 2mL of semen, post-ejaculate urine analysis evaluates is needed for retrograde ejaculation. Interpretation of the semen analysis relies on the andrologist's expertise. Total sperm count, rather than concentration, should be used for determination of oligozoospermia. Concentration does not account for volume and thus men with normozoospermia may have a low concentration and a high semen volume. Total motile sperm has been suggested as a better predictive parameter for male infertility. Sperm morphology, although included in the WHO classification, has been shown to have no impact on natural conception, IUI or IVF outcomes, and thus should not be used solely as a diagnostic criterion for male infertility. It is notable that while abnormal semen parameters can suggest a male factor, the presence of normal values does not guarantee fertility, nor does it rule out modifiable male factors.

Additional laboratory evaluation with a hormonal workup should include a determination of concentration of morning serum follicle-stimulating hormone (FSH) and testosterone, with or without an estradiol depending on the patient's body habitus/BMI. Luteinizing hormone (LH) and prolactin can be considered for men with low testosterone. Hypogonadotropic hypogonadism should be further evaluated with a prolactin level, and a pituitary MRI should be performed for an elevated prolactin level. Empirical medical management with anti-estrogenic compounds such as clomiphene citrate or anastrozole can be considered in patients with inappropriately normal FSH and low or borderline low testosterone levels. Genetic testing with a karyotype and Y chromosome microdeletion should be performed for patients with severe oligozoospermia or azoospermia. Latest Guidelines jointly published by the American Society of Reproductive Medicine and the American Urological Association recommend a cutoff of 5 million sperm/mL, but these authors' clinical practice is to test any patient with less than 5 million total sperm. Scrotal ultrasound and transrectal ultrasound should not routinely be performed. Scrotal ultrasound is reserved for patients with unexplained testicular asymmetry or palpable masses, as well as select patients with progressive decline in semen parameters. Transrectal ultrasound can be offered for patients with low volume, low pH semen azoospermia and palpable vasa. Diagnostic testis biopsy is not indicated in most patients with azoospermia, but can be used to determine the extent of spermatogenesis if the patient is counseled on its role in predicting sperm retrieval and desires further information prior to microsurgical testicular sperm extraction (microTESE).

Assessment of DNA fragmentation has become increasingly utilized, with terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) and sperm chromatin structure assay (SCSA) being the most widely accepted assays. Sperm DNA can be affected by free radicals and lead to poor sperm quality, affecting conception as well as increasing miscarriage rates. Instances in which DNA fragmentation testing may be useful include recurrent miscarriages, unexplained fertility, or multiple failed intrauterine insemination (IUI) cycles. Varicoceles are also known to cause DNA fragmentation, and evaluation of DFI can be useful in certain patients who have normal semen parameters and grade 2 or grade 3 clinical varicoceles. Microfluidic sperm sorting devices can be used in patients with a high DFI for in vitro fertilization (IVF) or IUI, with data suggesting it can improve clinical pregnancy rates.

The workup of male fertility requires a thorough history, physical examination and laboratory evaluation, While an etiology may not always be determined, it is important to have a fertility specialist involved for adequate counseling and treatment options. As genomic and proteomic tests emerge, new diagnostics and treatment options may follow.

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Chapter 33
Why evaluate the infertile male in the era of ART?

Medical and surgical therapies for male infertility

Marc 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 3 to 10 times higher incidence of testicular cancer in men with severe male factor infertility compared to age-matched controls.
2) There is a 30 to 100 times higher incidence of genetic abnormalities in infertile men. A majority of these abnormalities being Klinefelter syndrome (with 47 XXY karyotype)
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 of sperm, or non-obstructive, where the problem is lack of sperm production. In men with obstructive azoospermia, 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 dependent on female partner age, vary from 50 – 80%, depending on when the obstruction occured. 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 sperm aspiration from the vas, epididymis and testis, performing assays of sperm DNA integrity, e.g., a TUNEL assay. This allows the urologist to tell the andrologist which sperm source has the best DNA integrity, allowing a significant improvement in live birth rates compared to just using sperm morphology and motility to select the best sperm for ICSI.

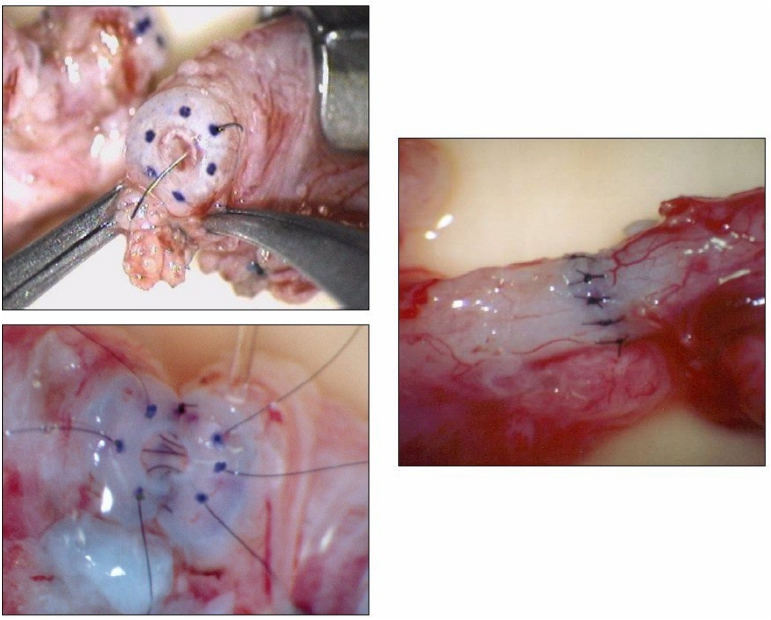

Figure 1. Surgical procedures involved in microsurgical vasovasostomy.

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, again using an assay of sperm DNA integrity to determine the best sperm source. The use of microfluidics to select sperm with dramatically better DNA integrity is also a promising new approach for some couples. Treatment of 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 penile 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, 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 naturally conceived pregnancy rates.

Klinefelter's Syndrome (47XXY) may present in a similar fashion, with the failure to go through puberty, and feminized habitus (Chapter 37). 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 cases, with a live birth rate of 30% in these men in whom sperm were found.

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 viable sperm in the ejaculate in about 40% of men. Empiric treatment of men with low serum testosterone levels, using either clomiphene citrate or the aromatase inhibitors anastrozole or testolactone, 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 naturally conceived pregnancy rates of over 40%. In addition, microsurgical varicocelectomy significantly increases testosterone levels and is already accepted as a treatment for androgen deficiency.

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

Prevention

The testis manufactures approximately 1,000 sperm every heartbeat and is regarded as the “canary” of the human body. It is exquisitely sensitive to the adverse effects of environmental

disrupters and gonadotoxins (Chapters 41-43). 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 (Chapters 24, 47). 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. Testosterone, an effective contraceptive for men, is to be avoided when future fertility is an issue. In androgen deficient men, unresponsive to other treatments, there is some evidence that treatment with testosterone plus clomiphene citrate and/or hCG can prevent the negative effect of exogenous testosterone on spermatogenesis.

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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Chapter 34
Can empiric medical treatments improve idiopathic male infertility?

Jared M. Bieniek and Kirk C. Lo

Infertility is a frustrating condition affecting 15% of couples with identifiable male factors contributing to half of cases. Evaluation of the infertile man includes a thorough medical history, focused physical examination, and initial laboratory testing including a semen analysis and reproductive hormones. Targeted medical management or surgical therapies may be offered for specific findings. Idiopathic infertility, defined as the presence of semen analysis abnormalities with no historical infertility risk factors, a normal physical examination, and normal endocrine testing, often proves more challenging.

The major components of a comprehensive semen analysis describing sperm quantity and quality include sperm concentration, motility, and morphology (sperm shape) (Chapter 22). Decreased sperm concentration, motility, or an increased number of abnormal forms can be seen in men with idiopathic male infertility (IMI). These abnormal findings may often present together. The prevalence of IMI among infertile men ranges between 30-40%.

Despite the frequent finding of IMI, treatment options remain limited. Recent American Urologic Association (AUA) and European Association of Urology (EAU) guidelines on male infertility both review supplements and various hormonal medications specific to IMI treatment. Extensive reviews also detail the status of many of these prescription and non-prescription options. This chapter summarizes the data and recommendations for empiric medical treatment of men presenting with IMI.

Supplements

Supplements as a class include a wide variety of vitamins, antioxidants, and nutritional supplements. Antioxidants in particular have been extensively studied for the treatment of male infertility given

the link between increased oxidative stress and male subfertility. Reactive oxygen species, which form as a result of oxidative stress, can impair sperm function and DNA integrity. Various single antioxidants or combination regimens have been reported including vitamin C, E, selenium, zinc, folic acid, carnitine, lycopene, and coenzyme Q10 among others.

The most recent 2019 Cochrane meta-analysis included 61 randomized controlled trials (RCTs) with over 6,000 men and concluded that antioxidant supplementation led to slightly improved pregnancy and live birth rates. However, many of the included studies were of low quality with no evidence of increased live birth rate when excluding studies with high risk of bias. The more recent Males, Antioxidants, and Infertility (MOXI) trial randomized 174 men with abnormal semen parameters to an antioxidant combination or placebo. Over six months of treatment, no significant changes in semen parameters, pregnancy, or live birth rate were found. Antioxidant use appears to be safe with no increase in miscarriages and low risk of gastrointestinal (GI) symptoms.

The use of supplements for treatment of male infertility is of "questionable clinical utility" based on available literature per the 2020 AUA & American Society of Reproductive Medicine guideline on male infertility. It is not possible to recommend any specific antioxidant formulation owing to the variability of reported regimens. The 2021 EAU guideline acknowledges the weak evidence supporting antioxidants in improving semen parameters in IMI but likewise does not make any formulation recommendations.

The heterogeneity and quality of studies on antioxidant supplementation make it challenging to draw conclusions for the clinical treatment of IMI. The availability, relative safety, and general low cost of supplements may make them an appropriate option in selected individuals with risk factors for oxidative stress. Antioxidants in higher-than-recommended doses should be avoided due to the risk of conversion to reductive stress, which can also be detrimental to sperm.

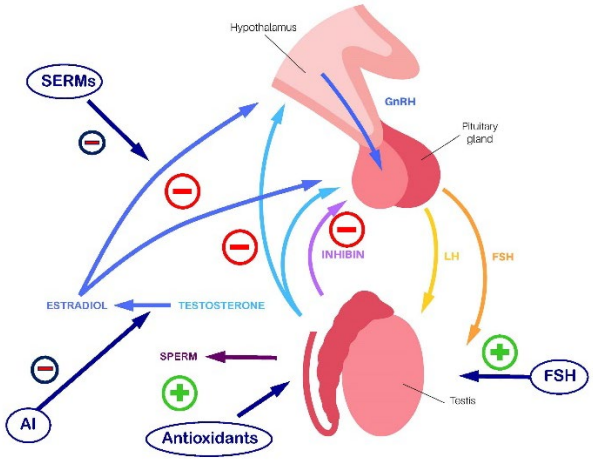

Figure 1: Hypothalamic-pituitary-gonadal axis with mechanisms of action of empiric male infertility treatments (dark blue text corresponds to medication or supplement class; AI = aromatase inhibitor, FSH = exogenous follicle stimulating hormone, SERMs = selective estrogen receptor modulators)

Selective Estrogen Receptor Modulators (SERMs)

Prescription medical treatments for male infertility target the hypothalamic-pituitary-gonadal (HPG) axis to increase signaling and downstream sperm production (Figure 1) (Chapter 2). Briefly, natural pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus leads to release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary. FSH in turn stimulates Sertoli cells which support spermatogenesis while LH acts on Leydig cells to cause testosterone production. Testosterone, estradiol, and inhibin, a protein secreted by Sertoli cells, provide negative feedback to the hypothalamus-pituitary axis to maintain homeostasis.

SERMs represent a class of medications that exert agonistic or antagonistic effects on estrogen receptors based on medication and tissue type. These medications are useful for treatment of male infertility due to their blockade of estrogen receptors in the hypothalamus and pituitary, thus increasing GnRH, gonadotropins, and driving sperm and intratesticular testosterone production (Figure 1). Clomiphene and tamoxifen are the most commonly selected

SERMs for treating male infertility though neither is approved for this use, thus they are considered off-label.

While the use of SERMs and other hormone stimulating medications is supported for the treatment of infertile men with low testosterone, the data is mixed among men with IMI who, by definition, have normal endocrine values. A meta-analysis in 2013 including 11 studies found significant improvements in sperm count and pregnancy rates in men with IMI treated with SERMs. However, the overall effect on pregnancy rate remained small with few studies reporting complications associated with SERMs that can include blurred vision, breast tenderness, and low risk of thromboembolism.

Again many of the individual studies included in the above reviews are low quality leading guidelines to conclude that SERMs offer limited benefit to men with IMI, providing minimal improvements to fertility outcomes and possibly delaying future treatments. While the goal of SERM use is to drive the HPG axis forward and improve semen parameters, the benefits may not be realized for men with IMI who already have normal endocrine values. Relative to empiric SERMs, assisted reproductive techniques (ART), such as intrauterine insemination or *in vitro* fertilization, offer greater fertility success and should be discussed as a part of shared decision making. SERMs remain a valuable tool in selected men with IMI, particularly those with borderline endocrine values or those who cannot pursue ART due to cost or religious reasons. Additional research may better define men with IMI who can derive the greatest benefits from empiric SERM administration.

Aromatase Inhibitors

Estradiol, present in low concentrations in men, is an important male hormone but when elevated can cause excessive negative feedback on the HPG axis (Figure 1). Multiple cell types, particularly adipocytes and Leydig cells, contain aromatase, the enzyme responsible for estradiol production via aromatization of testosterone. Increases in serum testosterone or adiposity can lead to an increased estradiol level, low testosterone/estradiol (T/E) ratio (defined as <10 when T is reported in ng/dL and E in pg/mL), and downstream effects on spermatogenesis.

Men with IMI have normal endocrine parameters but those with abnormally low T/E ratio may be logical candidates for empiric treatment with an aromatase inhibitor (AI). AIs inhibit

estradiol production and associated central HPG negative feedback, leading to increased hypothalamic and pituitary signaling and testicular function. AIs may also be combined with a SERM to reduce excessive aromatization of testosterone with estrogen receptor blockade. Commonly used AIs for male infertility include anastrazole and letrozole, both of which are off-label, much like SERMs.

Most trials evaluating empiric AI use include subfertile men with low testosterone, not truly IMI. As expected, a 2019 meta-analysis of eight such studies demonstrated significant improvements in endocrine and semen parameters. Pregnancy outcomes were not reported in the included trials. A low number of men (3.2%) stopped therapy due to side effects with no bone metabolism disorders identified. High-quality prospective studies are needed to evaluate the role of empiric AIs specifically in men with IMI.

The EAU male infertility guideline notes insufficient evidence to support AI use for treatment of IMI The AUA guideline recommends considering AI administration for infertile men with low testosterone levels but does not mention empiric AIs for IMI. The use of AIs for IMI may be appropriate for selected patients with abnormal or borderline T/E ratios who are not candidates for or wish to avoid ART. Potential adverse effects to discuss before prescribing include GI upset, decrease in libido, abnormal bone metabolism with chronic use, and low risk of thromboembolism.

Exogenous FSH

Exogenous FSH is approved only for use in men with hypogonadotropic hypogonadism and can be administered as a subcutaneous injection. Given in purified or recombinant forms, it acts directly at the testicular level on Sertoli cells to support sperm production (Figure 1). Exogenous supplementation in men with IMI having normal FSH has been extrapolated to likewise increase sperm count, quality, and fertility outcomes.

An updated 2013 Cochrane systematic review included six RCTs with 456 men comparing FSH treatment to placebo or no treatment for idiopathic subfertility. Despite varying protocols, exogenous FSH treatment resulted in higher pregnancy rates (16% vs 7%). No differences were found in a subgroup analysis of couples utilizing ART. A more recent 2015 meta-analysis reviewed 15 controlled trials comprised of over 1,200 men. Spontaneous

pregnancy rates were similarly improved with FSH use (11% vs 2%). Pregnancies after ART demonstrated a small but significant increase with FSH in this review. Optimal FSH dosing for IMI remains uncertain.

Available studies on FSH analogues for IMI led the EAU guideline committee to conclude, albeit weakly, that treatment may improve sperm concentration in men with low sperm concentration. The AUA guideline recommends considering exogenous FSH to improve sperm concentration and fertility outcomes but admits that the cost-effectiveness of treatment is questionable. In clinical practice, FSH analogues must be used for three months or longer with significant associated cost and limited improvements in pregnancy outcomes. Due to limited cost-effectiveness for IMI, shared decision making discussions are necessary before embarking on FSH treatment.

Summary

Despite reported improvements in semen parameters and fertility outcomes with empiric IMI treatments, the heterogeneity of studies makes it challenging to draw conclusive recommendations. Supplements, particularly antioxidants, offer a cost-effective option for men with IMI but the literature is not sufficient to support a specific formulation. SERMs and AIs may be considered for carefully selected individuals, particularly those wishing to avoid ART. The use of FSH analogues is supported by limited evidence but the cost, duration, and limited effectiveness of treatment challenge its practicality. Exogenous testosterone should always be avoided due to its suppressive effects on the HPG axis. Ultimately, IMI has limited empiric medical treatment options and ART should be an option in any treatment discussion, particularly for couples with a female partner age of 35 or greater.

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Chapter 35
What are the assisted reproductive technologies for male infertility?

Indications for IVF/ICSI/IUI, surgical sperm retrieval techniques

Peter 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, it was previously thought that 5 million motile sperm must be present in the ejaculate. More recent data from a large study showed that IUI success rates increased from about 7% pregnancy rate per treatment cycle when 1-2 million motile sperm were available after processing, up to about 15% when 5-7 million motile sperm or more were available for insemination. This limited procedure (requiring only sperm washing and insemination of the washed sample into the uterus) had reasonable cumulative pregnancy rates, but often required several treatment cycles to be successful.

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 3-5 days. This process may also allow for embryo biopsy (sampling of 3-5 cells for their genetic content), typically performed before embryos are frozen at the 5-day stage or later.

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 had abnormal morphology, then IVF was not very successful; only normally shaped sperm will naturally bind to the cumulus complex around the oocyte and fertilize. 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 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 oocyte fertilization and 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 sperm from semen samples with impaired motility, morphology or of limited number as well as "immature" sperm that are surgically retrieved from the male reproductive tract.

Men with azoospermia (a lack of sperm in the ejaculate) can commonly be treated with sperm retrieval and assisted reproduction with ICSI. Azoospermia can be due to a blockage/lack of development of the structures of the male reproductive tract (obstructive azoospermia) or from spermatogenic failure, where sperm production is so low that no sperm reach the ejaculate.

In obstructive azoospermia, sperm are produced and reabsorbed within the male reproductive tract, so the most viable sperm are often present closer to the testis than the point of obstruction (typically in the epididymis or vas deferens). These men have plentiful sperm production, so sperm retrieval from the testis or epididymis can be done with needle aspiration or biopsy. A larger number of sperm can be retrieved with a minor surgical procedure in the epididymis (microsurgical epididymal sperm aspiration), where more abundant sperm with better motility (and ability to be frozen for subsequent use in multiple aliquots) is possible than with testicular sperm retrieval.

In distinction, most men with non-obstructive azoospermia have either low levels of sperm production throughout multiple areas of tissue or focal areas of sperm production in isolated sections of the testis. For these men, sperm retrieval is most effectively accomplished with the microsurgical procedure referred to as microTESE (microsurgical testicular sperm extraction), where the surgeon uses an operating microscope to identify the focal areas with larger seminiferous tubules. The larger tubules contain more germ cells and therefore are more likely to contain sperm than the smaller tubules. Even men with conditions that severely limit sperm production such as Klinefelter syndrome (Chapter 37) can have sperm retrieved in 40-70% of cases. Since microTESE follows the natural anatomy of the testis and its blood supply, it is also safer than simple random biopsies of the testis.

So, applications of ICSI extend to patients with limited sperm number (oligozoospermia), severely impaired motility (asthenozoospermia), and sperm that are not normally shaped (teratozoospermia) possible to use for ICSI, as well as sperm surgically taken from the epididymis or testis that have not passed through the male reproductive system can also be used for ICSI. Most fertility centers now use ICSI for any IVF cycle when even marginal sperm abnormalities are present. The development of ICSI also encouraged us to consider treating men with azoospermia, 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 (non-obstructive azoospermia).

ICSI is only performed as an adjunctive procedure during IVF. Individual spermatozoa are selected based on their appearance and the presence of motility which confirms the viability of the spermatozoon to be used for injection. The sperm is manipulated to limit that motility (avoiding physical disruption of the oocyte after injection by a motile sperm). The oocyte is punctured by an injection pipette, and a single spermatozoon is injected per oocyte. This process allows fertilization and pregnancy with a very limited number of spermatozoa.

The fertilized oocytes (now embryos) are then 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 30-40%, but the chance of pregnancy when a woman is over 40 years of age drops to a level as low as 10% per initiated IVF cycle.

Since the sperm that are selected for IVF and ICSI would not have naturally fertilized eggs in the past, concern existed about the risk of potential birth defects after application of IVF/ICSI (Chapter 38). 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 increases from 0.2 to 0.8% with ICSI; this may occur because of abnormalities in the sperm provided by the man 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 able to be treated using IVF/ICSI who could not have naturally fathered children before. Male infertility can be managed with specific treatment of men, correcting hormone abnormalities, obstructive conditions in the male or repair of varicoceles, allowing for natural fertility. However, 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.

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Chapter 36
Genetic factors likely cause a large percentage of male infertility

Dolores J. Lamb

The genetic basis of male infertility probably represents one of the most important, yet under emphasized, cause 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) (Chapters 32, 33). 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 35). 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 37).

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. One such defect is a Robertsonian translocation that results when two different number chromosomes with a very long and a very short arm recombined together during the production of sperm or eggs. It is easy to see on a karyotype analysis. Individuals with this type of structural chromosome defect are at risk of infertility, pregnancy loss or having a child with a significant birth defect. Another important structural chromosome defect is a 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 oligozoospermic 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 while some of our knowledge comes from studies in animal models, today we have a much better understanding of the human genes impacting some forms of male infertility. 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 (Chapter 4), genes involved in structural aspects of spermatogenesis and cell-cell interactions, the formation and function of the sperm and fertilization can cause male infertility. While identifying specific defects in most infertile men remains difficult in individual patients, there are some areas where great

progress has been realized. 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. CBAVD patients who do not have cystic fibrosis also can have mutations in this 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 factor couple, both partners should be tested for mutations causing cystic fibrosis even if the spouse tests "negative" for *CFTR* mutation, 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. An additional gene that causes CBAVD has recently been identified, adhesion G protein coupled receptor G2 (*ADGRG2*), which causes an X-linked form and can be associated with unilateral renal agenesis.

Two major areas for which there is a significant increases in our understanding of the genetic basis of male infertility are poor sperm shape (morphology) and poor sperm motility. A subset of infertile men may have normal sperm concentration/density and motility but, for example, head defects where the sperm have a round head rather than being shaped somewhat like a tennis racket. This is because they may have a misplaced, atrophied, or absent acrosome. The acrosome resembles a "sock" over about 75% of the top of the sperm head which contains the enzymes needed to be liberated when the sperm has to traverse the egg investments to eventually penetrate the egg for fertilization (Chapter 25). Defects of two genes (among others), *DPY19L2* and *SPATA16*, can cause round-headed sperm. Other men have tail defects due to gene defects affecting the internal structures

within the sperm tail/flagellar and these genes encode proteins needed for subcellular sperm structures such as the outer and inner dense fibers, the dynein arms and other parts of the tail.

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 Beckwidth-Wiedemann. The majority of the children born seem healthy (Chapter 38). Obviously, long-term studies of the safety and efficacy of these procedures are required. There is a concern that some infertile men have health risks associated with their infertility. These men may have a higher risk of developing malignancies at early ages or have increased mortality and morbidity. Although the genetic or genomic defects linking infertility with health risks are not clearly defined, it is an area of intense investigation (Chapter 64).

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 is of critical importance.

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Chapter 37
What is Klinefelter Syndrome and how best to care for these patients

Samuel R. Donnenfeld and Akanksha Mehta

In 1942, Harry F. Klinefelter and colleagues published "Syndrome Characterized by Gynecomastia, Aspermatogenesis without A-Leydigism, and Increased Excretion of Follicle-Stimulating Hormone" in The Journal of Clinical Endocrinology & Metabolism, first describing what would later become known as Klinefelter's syndrome (KS). Klinefelter's Syndrome is the most common sex-chromosomal aneuploidy in males, characterized by a supernumerary X-chromosome, which affects 1:500 males. Phenotypic characteristics include but are not limited to cryptorchidism, hypospadias, small testes, intellectual disability, delayed or incomplete pubertal development with normal or low serum testosterone, and infertility. The predominant form of KS, which is present in 80–90% of cases, is defined by a 47, XXY karyotype whereas higher-grade aneuploidies (e.g. 48, XXXY or 48, XXYY), or mosaicisms (e.g. 47, XXY/46, XY) make up approximately 10–20 % of cases. Phenotype varies with severity of genotypic abnormality, which contributes to Klinefelter syndrome being under-diagnosed.

Laboratory examination of KS cases resembles that of primary testicular failure, and is typically characterized by non-obstructive azoospermia, low serum testosterone, and high serum LH and FSH levels. Importantly, men with KS, have higher rates of diabetes, epilepsy, cerebrovascular disease, breast cancer, and non-Hodgkin lymphoma, compared to euploid males. Lifespan for KS men is generally similar to that for euploid, men; however, life expectancy is 1-2.5 years less than the general population.

Early identification of individuals with KS is key for managing their underlying hormonal derangements. Management typically focuses on three major facets: gynecomastia, hypogonadism (and indirectly, fertility), and the psychosocial aspects of the disease which are often the most trying aspect of the disease for patients.

While treatment such as testosterone replacement therapy is an important aspect of care delivery, a multidisciplinary approach is recommended, given the fact that this disease affects multiple organ systems.

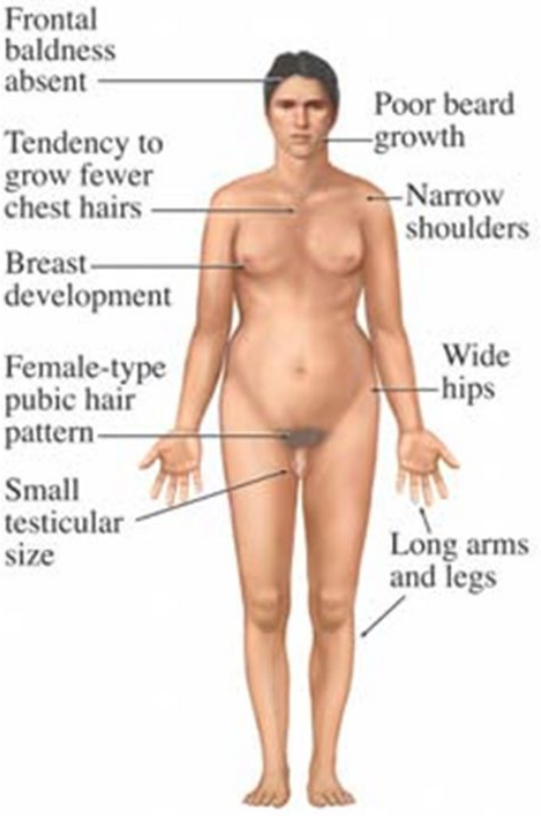

Figure 1. Klinefelter's syndrome.jpg. (2020, September 27). Wikimedia Commons, the free media repository. Retrieved 13:34, June 20, 2022 from https://commons.wikimedia.org/w/index.php?title=File:Klinefelter%27s_syndrome.jpg&oldid=473127011

Childhood Therapy and Prenatal Diagnostics

Only 25% of patients with KS are diagnosed with the disease in the pediatric age group. However, pre-natal diagnosis is becoming more common as an incidental finding on pre-natal testing such as amniocentesis and chorionic villi sampling, performed for other indications. In the post-natal period, karyotype analysis of a peripheral blood sample remains the primary diagnostic test for Klinefelter syndrome.

During mini-puberty, the sex-specific activation of the hypothalamic-pituitary-gonadal axis begins between 2-6 months of age. During this time, patients with micropenis or hypospadias can be treated with topical or intramuscular testosterone to promote maturation. Although this therapy has been utilized for the purpose of penile growth and development in infants with KS, data on long-term benefit are limited.

Many children with KS will present with cognitive and developmental abnormalities, including, but not limited to, hyperactivity, decreased ability to cope with labile emotional states, attention, and poor verbal comprehension. Patients exhibiting these symptoms should be referred to developmental psychologists and therapists with expertise in this area.

Puberty for KS patients tends to begin within the normal age ranges; however, some patients with KS do suffer from delayed puberty (Chapters 28, 35). In the majority of cases, once initiated, puberty does not progress in a timely fashion. As such, assessment of tanner stages, measurements of testosterone and gonadotropins, and body proportion measurements should be trended during pubertal years to determine the need for testosterone supplementation. In cases of delayed puberty and or symptoms of hypogonadism with corresponding lab values, testosterone supplementation may be considered. Testosterone replacement therapy is indicated to help patients progress through puberty if they are struggling with cognitive function or secondary sex characteristics. Suppression of spermatogenesis is a risk of testosterone supplementation and should be discussed with the patient. Long term impact of testosterone supplementation on fertility outcomes is unknown. Sperm banking should be offered to all patients prior to initiating testosterone supplementation.

During puberty, while spermatogenesis cannot be observed in the majority of seminiferous tubules, a minority will begin spermatogenesis and few spermatozoa can be found on microscopic

examination. In one 2016 study, adolescents with KS aged 13-14 years had spermatozoa collected in only 10% of the TESE (testicular sperm extraction) attempts, while in adolescents of 15-19 years, spermatozoa were found in 45%. Serum testosterone level does tend to correlate with prognosis of TESE success in identifying sperm. In the same 2016 retrospective study, Klinefelter's syndrome patients with spermatozoa in their ejaculate had similar mean T levels (10.2 ± 2.7; range 7.1–15.3 nmol/L) and similar mean LH levels (15.5 ± 7.1 U/L; range: 7.1–18.1), compared to those of patients with azoospermia (T: 12.3 ± 5.5 nmol/L; range: 2.3–31; LH: 19.3 ± 12.3 U/L; range: 1–62). However, in all nine spermatozoa-positive patients with KS, T levels were ≥7.0 nmol/L[i]. [I wonder if the authors would considered adding the values of testosterone in ng/dL for the US readership]

Infertility Management

Most men with KS will be azoospermic or severely oligospermic. According to the 2020 AUA/ASRM Guidelines, these men should be evaluated with karyotype and y chromosome microdeletion test to consider all possible underlying causes. Once KS is confirmed, these patients should be counseled about associated health risks, including an increased risk of breast cancer, osteopenia/osteoporosis, type 2 diabetes, and increased risk of thrombosis. An EKG should be done at least once during their lifetime to check for elongated QTc and risk of Torsades-de-pointes. Patients with or without gynecomastia should receive imaging with ultrasound or mammogram as well as a clinical breast exam at least every two years.

Sperm banking should be offered to every patient wishing to have children who carry a diagnosis of KS as well as any patient being considered for testosterone replacement therapy (TRT). In a study published in 2006, it was identified that KS patients' sperm counts can decline as they age with increased testicular atrophy and decreased spermatogenesis. Furthermore, all patients with KS and confirmed azoospermia as well as a current or future wish for paternity should undergo a testicular biopsy for TESE and be educated about possible benefits of microdissection-TESE (mTESE). Patients falling into this category should also be counseled on alternatives including use of donor sperm, adoption, or donor embryo utilization. While sperm retrieval rates in KS patients is lower than that of the population of non-obstructive azospermic patients, retrieval rates may be as high as 50% and have been used

via intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) to produce offspring.

Adult Hormonal Management

For adult KS patients testosterone supplementation is recommended in several patient groups. In the KS patient whose fertility issues have all been addressed, testosterone supplementation should be recommended if the patient has hypogonadism on laboratory results as this helps prevent certain aspects of the disease, namely those concerned with decreased bone mineral density. These patients should be monitored as every other hypogonadal patients on testosterone supplementation, with interval monitoring of hemoglobin and hematocrit levels and liver function studies every 6-12 months when on supplemental testosterone. Dose titration is recommended in accordance with response to therapy. In patients not receiving supplemental testosterone, it is still recommended that they receive an endocrine evaluation every 12 months. As with all patients with low testosterone, prior to offering testosterone therapy, clinicians should measure hemoglobin and hematocrit and inform patients regarding the increased risk of polycythemia. PSA should also be measured in men over 40 years of age prior to the initiation of testosterone.

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Chapter 38
What is known about the health of ART-conceived offspring?

Sarah R. Catford

Introduction

The use of assisted reproductive techniques (ART), especially intracytoplasmic sperm injection (ICSI) has steadily increased worldwide. Using these techniques, more than 8 million infants have been born so far. The application of ICSI has extended beyond its original purpose to overcome male factor infertility to include subfertile couples with non-male factor infertility, despite evidence that it offers no advantage over standard IVF. Two thirds of all IVF cycles worldwide now use ICSI. Given the prevalence of infertility and the widespread use of ART, understanding the possible adverse health effects is an important clinical and public health issue.

The importance of evaluating health outcomes in ART-conceived children is supported by the knowledge that adverse periconception exposures influence offspring health and chronic disease risk. ART is associated with epigenetic changes that affect germ cells and embryo development, and these may be responsible for chronic diseases in adulthood. Ovarian stimulation and embryo culture are examples of ART procedures that have been implicated in the epigenetic changes in offspring. The ICSI procedure may pose additional threats, related to its more invasive nature. At this stage, it remains to be seen whether epigenetic changes linked to ART result in long-term health consequences.

The health of ART-conceived offspring is also influenced by parental characteristics affecting gamete quality and fertility, as well as epigenetic modifications arising from underlying parental infertility. In the context of male infertility, higher rates of chromosomal abnormalities, sperm DNA damage and sperm aneuploidy are observed in infertile men compared to fertile men. There is also a strong and complex genetic basis to the aetiology of male infertility. As such, the use of ICSI in cases of male factor infertility presents additional concerns including the heritability of male infertility and the effects of using poor quality sperm on other aspects of offspring health.

Health outcomes in ART-conceived offspring

Perinatal health and congenital malformations

Both IVF and ICSI are associated with an increased risk of adverse perinatal outcomes such as preterm delivery, low birthweight, multiple births and maternal complications compared to natural conception. The literature also shows an increased risk of congenital malformations in children conceived with IVF or ICSI compared to those conceived naturally, although the absolute risk remains low. ICSI-conceived children do not appear to be at greater risk of congenital malformations compared to children conceived with IVF.

Genetic conditions

Male and female infertility may be caused by underlying genetic defects, that prior to ART would not have been transmitted to offspring. The genetic origin of male infertility is particularly complex with more than 8000 genes required for spermatogenesis. An array of known genetic causes such as chromosomal rearrangements, y chromosome microdeletions and monogenic mutations explain 15% of male infertility; but the number of newly identified causative gene mutations is continually growing and a genetic cause probably explains a substantial proportion of male infertility overall. With the advent of ART and particularly ICSI, certain genetic variants may now be transmitted to offspring. Examples include mutations in the cystic fibrosis transmembrane regulator gene which cause cystic fibrosis and congenital bilateral absence of the vas deferens, and Y chromosome microdeletions that are inevitably passed onto sons. Other than specific gene mutations such as these, ART-conceived children are also at greater risk of imprinting disorders that are more common than in children conceived naturally.

Neurodevelopment

Many studies have focused on neurodevelopment during infancy and childhood with reassuring results. A substantial amount of good quality evidence now indicates that IVF- and ICSI-conceived children develop normally and have similar cognitive and motor performance, behaviour and family relations to their naturally conceived peers. There remains some debate about the risk of autism, as some studies have suggested a higher prevalence among ART-conceived children, but a recent large longitudinal study found

that children conceived by ART including ICSI were not at increased risk of autistic spectrum disorders compared to naturally conceived children.

General physical health

Data on general physical health during childhood, such as illnesses, hospital admissions and risk of surgical interventions are mixed and at least partially rely on parental reporting making it difficult to draw firm conclusions. Preliminary research involving ART-conceived adolescents and young adults suggested some physiological differences in health (e.g. higher blood pressure) compared to naturally conceived controls. However, the largest study of ART-conceived adults to date found no evidence of increased cardiovascular risk or growth or respiratory problems in 193 ART-conceived adults compared with 86 naturally conceived participants. Their psychosocial outcomes also appear similar with most ART-conceived children developing into healthy young adults. Two recent European population registry-based cohort studies with a median follow-up of 11 and 21 years found that the overall cancer risk was not increased in ART-conceived children compared to children conceived naturally, including those conceived by ICSI.

Metabolic health

The metabolic health of ART-conceived offspring has attracted attention due to recognition that poor metabolic health in adulthood is partly determined by early life events in utero. Studies in ART-conceived mice have shown higher blood pressure (BP), altered glucose metabolism, and endothelial dysfunction compared to naturally conceived mice. Various studies have shown poorer cardiovascular and metabolic profiles among IVF- and ICSI-conceived offspring compared to naturally conceived children, such as higher BP, elevated fasting glucose, insulin resistance and disturbed body fat composition. A recent meta-analysis showed a minor, yet significantly higher BP in 872 IVF/ICSI-conceived offspring compared to 3,034 naturally conceived controls. The previously mentioned study of ART-conceived adults, however, found no evidence of increased vascular or metabolic risk compared to naturally conceived controls, as indicated by carotid artery intima-media thickness, pulse wave velocity, BP, serum metabolic markers and anthropometric measurements. There is too little data on the metabolic health of ICSI-conceived adults to speculate, but current data suggest it is equivalent to naturally conceived

individuals. Large prospective longitudinal studies with more sophisticated measures of metabolic risk, such as 24hr ambulatory BP monitoring and glucose clamp studies, are needed to definitively assess this risk.

Reproductive health

Many studies have evaluated the reproductive health of ICSI-conceived offspring during adolescence with data suggesting similar pubertal development compared to naturally conceived adolescents as assessed by Tanner staging, age at menarche and reproductive hormone levels. Data on the reproductive health of IVF-conceived offspring and IVF- and ICSI-conceived adults, however, are sparse. Consistent with longstanding concerns about the use of ICSI, a Belgian study published in 2016 suggested ICSI-conceived men may experience poorer reproductive health compared to their naturally conceived peers due to findings of reduced sperm quantity and quality. This study was limited by small sample size, lack of information on aetiology of paternal infertility and likely participation bias in the naturally conceived controls.

A larger Australian study published in 2022 found no differences in sperm output and serum FSH levels between a cohort of 120 ICSI-conceived men and a population representative sample of 356 naturally conceived men. In this study, ICSI-conceived men had slightly lower mean progressive sperm motility than naturally conceived controls, but mean values were still above the reference limit. The clinical significance of such a minor difference is unclear. This latter study also compared a subgroup of ICSI-conceived men whose fathers had spermatogenic failure to naturally conceived men, as well as to ICSI-conceived men whose fathers had obstruction (e.g. prior vasectomy), to examine the effect of paternal infertility. It was hypothesised that sons of fathers with spermatogenic failure may be at greater risk of poor reproductive health, given its strong genetic origin, than those of fathers who had an obstructive defect and naturally conceived men. The same findings were observed between ICSI-conceived men of fathers with spermatogenic failure and naturally conceived controls, as was the case for the entire ICSI-conceived cohort. Interestingly, reproductive parameters were similar between ICSI-conceived men of fathers with spermatogenic failure and those of fathers with obstruction; however, the size of the latter subgroup was small. Overall, these results suggest that health differences may relate to the IVF/ICSI procedure, rather than underlying paternal spermatogenic failure. Further research is

required to clarify these findings and ultimately data on the ability of ICSI-conceived men to achieve pregnancy and produce live births are needed.

Mechanisms for potential adverse health outcomes

So far, it is not possible to determine whether potential differences in health outcomes are due to the ART procedure, complications associated with ART such as prematurity, or heritable parental characteristics. A few studies have explored these potential contributions by including a control group of naturally conceived children born to subfertile couples and shown that the ART technique could be at least partly responsible for any ill health effects in offspring. This is supported by other studies showing that developmental outcomes are not worse in children of fathers with severe sperm abnormalities. However, methodological limitations of published studies, such as small sample size, inadequate control groups, high nonparticipation rate, subject heterogeneity and the complexity of ART treatment, make it difficult to isolate these effects.

Challenges of research in this field

There are numerous challenges of research in this area that explain the varying quality of available studies and the difficulty in providing definitive answers. These include (i) the requirement for multiple data sources to determine health outcomes; (ii) a preference for longitudinal information; (iii) sourcing of appropriate control groups; (iv) adjustment for multiple confounding factors; (v) consideration of technological changes in the ART procedures over time and between countries; (vi) attention to multiple potential sources of bias; and (vii) replication and validation of health outcomes in numerous studies.

Conclusions and future research

Whilst many health outcomes during childhood and early adulthood are likely to be comparable between ART-conceived and naturally conceived offspring, data on the long-term health of ART-conceived adults is still inconclusive. Interpreting existing data is difficult due to differences in methodology, variation in definitions and reporting of health outcomes and inconsistent control groups. Furthermore, with all of these health outcomes, it is difficult to determine whether

they relate to the procedure itself, complications arising from the procedure or to parental characteristics such as the infertility per se.

Given the increasing use of ART, especially ICSI it is essential that research continues. It is important that follow-up of ART-conceived offspring is continued into late adulthood, as the consequences of ART conception may not become apparent until later in life. Large well-controlled prospective longitudinal studies will be required to define health differences and determine their clinical significance. It is crucial that further research efforts focus on untangling the contribution of parental characteristics and factors related to ART. This will require the inclusion of several control groups including naturally conceived offspring from subfertile parents and those from age-matched fertile parents. Improved understanding of the health implications of ART conception will enhance couple counselling, improve clinical practice and aid related areas of reproductive research.

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Chapter 39
What should I know about artificial insemination of animals? How is male fertility assessed in domestic animals and why is this important?

Peter Sutovsky and Lauren E. Hamilton

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

Artificial insemination (AI) is a method of impregnating a reproductively receptive (in heat), presumed fertile female with semen from a presumed fertile male of the same species collected by means of masturbation (rubber glove method), artificial vagina collection or electroejaculation. In cattle, a cryopreserved plastic straw with 20 million spermatozoa (standard AI dose) is thawed at a controlled temperature and emptied into the uterus of a cow/heifer with the help of a stainless steel pipette (AI gun) manipulated through the cervix by the inseminator's arm inserted in rectum (recto-vaginal insemination process). In pigs, the plastic, lubricated insemination catheter is attached to a plastic bag with extended (diluted) fresh boar semen and a dose of 1-3 billion spermatozoa, often pooled from 2-3 boars, are deposited deep in the cervix without guidance by palpation. Boar semen is notoriously difficult to freeze without damaging spermatozoa, for which reason most swine AI is performed with fresh, cooled semen. Variations of these procedures are used in other livestock species, and most AI in horses, dogs and turkeys is done with liquid semen.

Legends of the first AI involve horse thieves impregnating mares by inserting stallion semen-soaked sponges go back a millennium, while the first scientifically documented AI was done in 18th century in dogs, by Italian priest Lazzaro Spallanzani, considered the father of fertilization biology. Spallanzani was also credited with the first "test tube" fertilization experiments by using semen collected from male frogs and dispersed over female frog eggs. Livestock/domestic animal AI in a modern sense entered the scene in late 19th and early 20th century, and reached true

commercial application after WWII, coinciding with the development of semen dilutants (extenders) and cryoprotectants such as glycerol (a polyol compound extracted from plant and animal sources) for bull semen preservation. The most recent major advance in AI has been the development and commercialization of AI with sexed semen, i.e., semen separated to carry sex chromosome of the desired gender, either female (X) or male (Y), which ultimately predetermines offspring gender at birth.

To date, AI has been used on a worldwide commercial scale in food animals such as cattle, pigs, goats, sheep and camels, as well as in pets (dogs, cats, horses) and trophy game (elk, deer). AI is also being developed for food animals of regional importance (e.g., guinea pigs in Central/South America, ostriches in South Africa), and for conservation purpose in captive wildlife animals and rare livestock breeds (e.g., large cats, rare cattle and donkey breeds). AI has been used in human ART (assisted reproductive therapy/infertility treatment) for centuries (first recorded insemination in 1700s by Scottish-born surgeon John Hunter), long before the first successful human in vitro fertilization (IVF; 1978); it remains a front-line treatment in treatment-seeking couples that produce gametes (spermatozoa and eggs) of acceptable quality and quantity, before IVF is considered. At present time, about 33 million bull semen units are sold in the USA annually, with an additional 39 million units exported. Over 34 million pigs are inseminated annually in North America, with the use of swine AI over natural breeding approaching 100%. Bulls are first collected at around 10-12 months of age and remain in service for an average of 3 years. Boars are typically collected at around 7 months and replaced after one year of service.

In the modern era of precision agriculture, the major advantage of livestock AI is the ability to amplify offspring of males with superior genetic makeup favoring desirable production traits such as meat or milk production, or ease of calving in cattle. Another major advantage is the ability to limit the spread of infectious diseases that can occur by mating, and to eliminate the labor and costs associated with maintaining a large number of male genetic stock and managing the breeding herds. Also reduced are injuries to animal handlers and to animals themselves that are associated with natural breeding. Optimized AI, hand-in-hand with precise female estrus detection and synchronization (timed AI) is a powerful tool for maximizing the benefit of genomic selection while also maintaining a reasonable level of heterosis (hybrid vigor) in livestock herds.

How is male fertility and AI semen quality assessed in livestock animals? Why is this important?

Fertility of an AI dairy bull is expressed as sire conception rate (SCR, measured by female non-return to estrus) while boar fertility is expressed by both the pregnancy rate and litter size. Pregnancy success in beef cattle is generally determined by ultrasound evaluation or rectal palpation of the uterus. All of these measures are critically influenced by andrological health. The first step in the andrological evaluation of a male animal is the general physical examination (body condition, eye, foot and leg problems), as well as visual assessment and palpation of external genitals and accessory glands. In yearling bulls intended for AI service, this is done as part of breeding soundness examination (BSE), which also includes measurement of scrotal circumference (an informative parameter associated with normal sperm production) and light microscopic semen analysis. A major limitation of the bull BSE is that it is a pass/fail exam and is a better indicator of infertility than fertility. Although the first fertility test for (human) couples was described by female physician Trotta of Salerno in 11th century, the first observation of human spermatozoa by Van Leeuwenhoek and Hamm occurred in 17th century and the modern, microscopy based andrological semen analysis came to existence in late 19th century. Thus, semen quality for male fertility evaluation and AI quality control has traditionally been performed by subjective light microscopic analysis including sperm count (total sperm number in ejaculate), concentration (sperm number per milliliter of ejaculate), motility (movement including speed and direction), percent of visually normal spermatozoa (normal sperm morphology), and the presence of immature sperm forms and non-sperm contaminants (bacteria, white blood and epithelial cells); it remains the standard method of semen analysis. Such assessments are done on a small aliquot of fresh semen shortly after collection and, for cryopreserved semen also after thawing one dose from a single collection batch prior to AI. Visually, collected semen can be assessed for volume, density (opacity related to sperm concentration), color (may identify blood, urine or purulent exudate contamination) and swirl (liquid semen streaming caused by sperm motility). Due to inherent subjectivity and inter- and intra-laboratory evaluator differences, automated, objective measurements of semen/sperm quality have been developed, based on bright field microscopy image/video output capture by digital cameras and analysis by

dedicated computer software (CASA/computer-assisted semen analysis). To increase the speed, cell number and accuracy, methods based on flow cytometry techniques have been developed that utilize tagging and bulk measurement of sperm samples with fluorescent probes reflective of sperm structural integrity, viability and fertilizing ability. Such measurements appear to be superior to conventional and CASA analysis in their ability to accurately assess the percentage of normal spermatozoa and even predict fertility of a semen dose.

What is the difference between subjective and biomarker-based semen analysis?

Light microscopic semen analysis provides useful baseline assessment of a male's ability to produce spermatozoa in sufficient quantity and with acceptable concentration, viability and quality. In extreme cases, such evaluations identify transiently infertile males or males with chronic infertility due to disease, genetic/inherited factors, malnutrition or environmental effects (heat stress, pollution), separately or together causing reduction or cessation of semen production/quality. In males with acceptable semen and sperm parameters, such analysis provides clues as to what the minimal number of spermatozoa needs to be in an insemination dose, an important consideration with regard to cost effective production of doses from males with high genetic value that in some cases need to compensate for increased content of defective spermatozoa in semen. Automated, objective andrological methods bring semen analysis to the next level, with its speed (thousands of spermatozoa per sample measured in a matter of seconds) and application of specific biomarkers potentially reflective of current and/or predictive of a future sires' fertility in AI service. Thus, objective semen assessment could supersede the microscopic portion of BSE and eliminate need for retesting. The next step in the development of andrological analysis involves artificial intelligence (bringing AI to AI), which will facilitate fully automated, label-free (no fluorescent probes required) sperm quality assessment.

Can semen be improved? Supplementation, purification and ultimately, sexing.

Ultimately, the goal of the livestock industry is to improve the AI semen dose by clinical, nutritional and environmental management

of the male, as well as by the optimization of protocols and media for semen processing, storage, distribution and deposition. This can be done indirectly by nutritional supplementation or by direct alteration of semen extenders with antioxidants, to scavenge semen's intrinsic reactive oxygen species harmful to spermatozoa, and naturally occurring antimicrobials reducing the need for antibiotics to prevent bacterial growth and disease transmission. An additional level of improvement can be attained by semen purification, removing defective and dead/moribund spermatozoa using centrifugation, sedimentation, filtration or magnetic/nanoparticle-based purification. Flow cytometric semen sexing/sorting could be viewed as an ultimate purification technique; in addition to removal of spermatozoa carrying the undesired gender of sex chromosome, it also discards dead spermatozoa, spermatozoa with abnormal chromosome complement (aneuploidy) and those with defects of DNA packaging. However, sexing of spermatozoa is a very slow procedure and thus AI doses are prepared with 10 to 20% of the spermatozoa in traditional semen dose. Procedures that remove subfertile or dead spermatozoa before sex sorting may be valuable.

Summary

Artificial insemination revolutionized animal production in the 20th century. At present time, it remains the leading method of reproductive management in pigs and dairy cattle in the US and worldwide, with its adoption increasing in other livestock species. Precision agriculture approaches of genomic selection and gender determination/semen sexing would not be possible without AI. Application of biomarker-based, automated semen analysis coupled with artificial intelligence/machine learning will further expand the use and precision of livestock semen analysis for fertility determination and AI semen quality control. Semen dose improvements are being made through feeding of male-optimized, balanced diets, nanotechnology, and judicious semen extender supplementation with sperm friendly additives such as antioxidants and naturally occurring antimicrobials. Altogether, such improvements will increase cost efficiency and reduce the number of males needed for AI service, thus maintaining profitability and reducing the environmental impact of livestock operations on air, water and soil.

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Chapter 40
Are sperm counts declining with time, to what extent and what are the consequences of this decline?

Géraldine Delbès

Since the mid-1970s, there has been continuing global concern regarding the potential for diminishing human reproductive health in industrialized countries. This issue has arisen in part in the context of the revision of minimum semen quality standards and some observations of declining sperm concentration over time in men. These reports have led to the worry that “something has altered the fertile male population to depress the semen quality remarkably"(Nelson & Bunge, 1974, p. 507). The hypothesis of sperm decline was further 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 20 different countries; an approximately 50% decline in sperm counts (113 to 66 million/ml) was found over that time frame. Additional concern about a temporal (secular) decline in semen quality was heightened by a 1995 French publication that reported a 30% decrease in sperm concentration (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 concentration, was in fact changing. the scientific community raised substantive questions about the validity of the data. 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.

In 2017, Levine et al. re-evaluated the question, addressing many of the above-mentioned methodological criticisms in a meta-analysis of 185 studies of human semen quality published between 1981 and 2013, representing 42,935 men from 50 different countries, who provided semen samples between 1973 and 2011. Using a meta-regression model, they reported a decline of 0.75% per year in sperm concentration and total sperm count for all men. This decline was the most pronounced in unselected men from Western countries (North America, Europe, Australia, and New Zealand) with 1.4% per year in sperm concentration and 1.6% per year in total sperm count. A similar but less steep negative slope was observed for selected fertile Western men. Overall, 59.3% decline in total sperm count (337.5 to 137.5 million) was found between 1973 and 2011 in the unselected western population. Interestingly, when separating data for other countries (South America, Asia and Africa) no significant trend were seen either in the unselected population or fertile men. Yet, since this meta-analysis, systematic reviews of Chinese, African or Indian populations, among many others, have also reported a decline in sperm concentration over time, with an approximately similar trend of ~1% decline per year. In 2022, a worldwide meta-analysis has validated these findings

The concordance of several strong meta-analyses reinforces and rally the scientific community on the fact that the sperm count is decreasing. Interpretation of the consequences yet remains. The media has seized on these data to conclude that there is a decrease in male fertility, and to question the causes. Indeed, sperm concentration is one of the determinants of fertility of an individual. According to the reference criteria established in 2021 by the WHO, the threshold of 10 million spermatozoa per ml can define infertility (Chapters 22, 32). The significance of the conclusion of a decline in sperm count worldwide therefore appears to be an alarm signal for the reproduction and survival of the human species. Concerns that environmental toxicants such as endocrine disrupting chemicals, as well as obesity, diet 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 increasing incidence of testicular cancer, and

congenital genito-urinary abnormalities (hypospadias, 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 led to the concept of a "Testicular Dysgenesis Syndrome" that might include downstream changes in semen parameters (Chapter 44). In short, this hypothesis suggests that alteration of the *in-utero* environment may be the common cause of these male reproductive abnormalities, leading to an overall decrease in human fertility.

Recently, Boulicault *et al.* presented the "sperm count bioavailability" hypothesis providing an alternative, more inclusive, framework for interpreting the global sperm count decline as described by the Levine report from 2017. This framework is based on the following principles: 1) above the WHO threshold, a high average sperm count is not necessarily optimal; 2) there is no species-typical reference in the 1970s; 3) semen parameters vary considerably within an individual or a population because ejaculate is an excretory product that is influenced by many external conditions without necessarily being associated with fertility or health hazard. While it does not rule out the possibility that the decline may have implications for population fertility, this analysis puts into perspective the significance of the overall decline from "normal" to "normal" sperm concentration by WHO criteria, suggesting that these trends may be due to benign or adaptive variation depending on a variety of individual and most of all geographic factors.

Based on the above discussion, it can be concluded at this time that there is a global temporal downward variation in human sperm count. However, the causes of this variation and its significance in terms of male fertility and men's health remain debatable. Indeed, regional differences in sperm count have not yet been explained and deserve further study. Moreover, the assumption that this decline will continue linearly over time remains hypothetical. The recent analysis of more contemporary data from 2000 onward suggests that the downward slope is getting steeper. In addition, the contribution of environmental causes including lifestyle changes and xenobiotics is suspected and strongly supported by experimental studies, yet, it still requires precise longitudinal prospective epidemiological studies. Because of the variability of semen analysis parameters, the statistical power of these studies requires large cohorts controlling for multiple confounding factors

making them difficult to do. Yet, these studies are heavilly needed to better understand the causes of sperm count decrease in Human and to determine the actual consequences on male fertility.

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Chapter 41
Do environmental contaminants including endocrine disruptors impair human sperm production and fertility?

Identification and regulation of man-made chemicals that may have adverse reproductive effects in men

Sally Perreault Darney

What is the evidence that environmental contaminants may alter sperm production and function in humans?

Back in the 1970s, a time when environmental risks to reproduction focused largely on females, 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 occupational health investigations found an association between the men's job-related 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 chemical exposures may be harmful for men as well as pregnant women.

Subsequent studies in small cohorts of men exposed to relatively high levels of chemicals in occupational settings, such as agricultural or industry workers, or via accidental chemical releases, raised the level of interest in male reproductive hazards in the workplace, motivating the National Institute of Occupational Safety and Health to conduct and fund male-specific research. Since then, occupational health studies have provided strong evidence that men exposed to a variety of workplace chemicals, including certain industrial solvents, pesticides, plasticizers, surfactants (e.g., stain repellants), flame retardants, and metals (notably lead and cadmium) can be at increased risk of lower sperm counts and/or semen quality.

In contrast, it has been more difficult to study and find convincing evidence of adverse effects in men from the general population where exposures to related environmental contaminants in air, water and/or food are typically at low levels relative to occupational exposures and are often accompanied by the simultaneous presence of other contaminants in the environment. The challenge of attributing abnormal semen quality to any specific environmental exposure is confounded by many other factors that can affect semen quality adversely. These include the man's age, body mass index (BMI), stress levels, and/or habits such as smoking or drinking alcohol. Cohort and population-based studies designed to detect associations between an environmental exposure of concern and a male reproductive outcome must also consider the timing and duration of the exposure, and the nature of the contaminant. For example, it may be easier to detect the effects of a chemical that bioaccumulates over time, such as the pesticide DDT, than a chemical the is rapidly metabolized such as Bisphenol A (BPA). Thus, evidence from studies in human populations, while highly relevant, is often inconclusive, making it of limited use for supporting regulatory actions.

Then how do regulatory agencies evaluate the safety of chemicals and other environmental contaminants with respect to potential male-specific reproductive effects?

The US Environmental Protection Agency (EPA), established in 1970, is charged with protecting human health and the environment by enforcing environmental laws enacted by Congress. Pioneering environmental laws passed in the 1970s and updated since then, include the Toxic Substances Control Act, the Federal Insecticide, Fungicide and Rodenticide Act, the Safe Drinking Water Act, the Food Quality and Protection Act, and the Clean Air Act. These laws, and similar laws in other countries, require industry to test chemicals for toxicity to ensure their safety. It is then largely up to the States and other local governments to enforce these rules. Although some chemicals were initially grand-fathered-in for use (meaning they did not need to be tested for toxicity), recent updates in the US laws now require that all new and existing chemicals must pass tests for safety before they are registered and approved for sale, distribution, and use, or approved for new uses (https://www.epa.gov/assessing-and-

managing-chemicals-under-tsca/frank-r-lautenberg-chemical-safety-21st-century-act).

Early protocols designed to test for adverse reproductive effects (male and female combined) involved exposing groups of experiment animals (rats or mice) continuously to several dose levels of the chemical across 2 or more generations. In these so-called Multigenerational Test designs, young adult male and female animals are exposed for at least one spermatogenic cycle and then mated. The production of litters is measured, including the number and normalcy of the pups. The pups are then dosed through puberty and mated again to assess fertility and litter size and quality. This traditional approach was improved in 1998 to include more specific measures of male reproductive function such as diagnostic histology of the testis, sperm concentrations in the testis and epididymis, sperm motility and sperm morphology, the latter enabling more direct comparison with human studies based on semen quality (http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health Effects_Test_Guidelines/Series/870-3800.pdf). These guidelines have now been harmonized across federal agencies in the US and internationally through collaborations with the Organization for Economic Cooperation and Development (OECD) whose members include Canada, Mexico, most of Europe, Japan, Korea, Israel and Australia.

Even so, it is now widely accepted that the multigenerational test approach has a number of serious limitations:

- These protocols are expensive to conduct as well as time-consuming (months at least) and are therefore not feasible for screening the large number of chemicals in production or use today and the thousands of new chemicals requiring safety evaluations each year;
- They require the use of many animals resulting in ethical concerns;
- They are limited in sensitivity because they typically include only 3 dosages (high with general toxicity, medium, and low with little or no effect) making them ill-suited for defining dose response at environmentally relevant concentrations;
- They provide little if any mechanistic information that could inform risk assessment and/or be extrapolated to related chemicals;
- Because they expose animals for an entire reproductive cycle, they may detect only apical endpoints without providing

information about the initial cellular targets or biological mechanisms that might inform assessment of other similar chemicals;
- Importantly, they are not designed to detect so-called "endocrine disruptor chemicals" or EDCs which may act at very low exposure levels to perturb reproduction via hormone-mediated modes of action. Exposures to EDCs during embryo-fetal development and early postnatal development can alter reproductive tract development and thereby increase the risk of infertility later in life (after puberty) or increase risks for certain reproductive tract cancers.

Recognizing these limitations, and the potential benefits of using new information from genomics, epigenomics, bioinformatics, metabolomics and systems biology to advance the field of toxicology, the research and risk assessment offices of the EPA, Canada and other countries and in partnership with OECD launched a revolution in toxicity testing in the early 2000s called "Toxicity Testing in the 21st Century." This effort has generated and continues to develop and evaluate New Alternative Methodologies (NAMs) for toxicity screening, chemical prioritization, testing, and risk assessment to meet the need for better and more efficient chemical evaluation, including tests that are specific to male reproductive health.

What are NAMs and how can they be targeted to detect and characterize risks of chemicals specific to male reproduction?

NAMs are an evolving collection of innovative in silico, molecular, in vitro, and short-term in vivo tests and computational approaches designed to enable more rapid identification of potential health risks of chemicals and/or to prioritize those with the greatest potential for harm for further testing. With NAMs risk assessors and toxicologists are provided with new ways to evaluate the health effects of chemicals that are more efficient and more mechanistically driven. Rather than first testing chemicals in animals, chemicals are prioritized and grouped based on what is already known about their chemical structure or biological activity and then tested using biochemical, molecular or in vitro tests that screen them for their ability to act as an initiator of, or trigger for, a biological change or disruption. Changes in cells can then be linked to changes in whole

organisms that are indicative of reproductive dysfunction such as decreased sperm production by the testis. Adverse Outcome Pathways are constructed over time, and form the basis for further, more organ- or system-specific testing.

The EPA launched its EDC screening and testing program to develop and validate NAMs specific to identifying chemicals with endocrine activity (https://www.epa.gov/endo), specifically activity that disrupts estrogen, androgen, or thyroid action. This effort is being extended globally in partnership with OECD. Of relevance to male reproductive toxicity, chemicals with EDC activity can now be identified using in vitro androgen receptor binding and transactivation assays, and/or cell lines that synthesize testosterone in vitro. With such assays, hundreds if not thousands of chemicals can be screened in short order to prioritize positives for further testing in animals.

Furthermore, this program validated short term animal tests that can now be used to confirm results of in vitro screening. For male reproductive toxicity, the Hershberger Assay, for example, uses immature (prepubescent) male rats with undeveloped prostate glands.Control rats are given exogenous testosterone that causes the prostate gland to grow; increased prostate weight is indicative of a positive response within only a few days. Test animals are given suspected androgenic chemicals and their prostate weights compared with the testosterone-stimulated control weights. Conversely, a chemical with anti-androgen activity can be identified by its ability to block this response when administered together with testosterone. Thus, this test is useful for detecting chemicals with either androgenic or anti-androgenic activity. Finally, chemicals positive in in vitro screening and/or the short-term in vivo tests can then be moved directly into a risk assessment to support regulatory decision making. Thus, these new approaches are not only more rapid than multigenerational tests, but they use fewer animals and provide mechanistic information for risk assessment.

How might chemicals impact male fertility via endocrine disruption?

The reproductive system is exquisitely sensitive to disruption by EDCs during critical developmental windows. After puberty, the male reproductive system continues to depend on optimal levels of androgens and estrogens, and can therefore be disrupted by exposures to environmental anti-androgens and/or estrogens,

albeit usually requiring higher levels than those known to act in utero. For example, the later stages of spermiogenesis depend on androgen, and there is increasing evidence for a role for estrogen in sperm transport and function. At environmental levels of these contaminants, decrements in sperm production, transport, or function may be subtle and difficult to detect. However, even subtle effects may exacerbate reproductive problems in men if they are already subfertile for other reasons.

Toxicology studies using both traditional and NAM-based testing have shown that the fungicide vinclozolin (and its metabolites) inhibits androgen action by binding to the androgen receptor. Some phthalates interfere with steroidogenic pathways resulting in reduced testosterone levels. If women who are pregnant with male fetuses are exposed during critical windows of reproductive system development, their babies may be at increased risk of being born with hypospadias (incomplete closure of the penile urethra) or testicular maldescent (both repairable with surgery) (Chapter 44). In adults, higher exposures to EDCs may also inhibit androgen biosynthesis or receptor binding and thereby dampen testosterone-dependent processes including sperm maturation with potential effects on fertility. EDCs may diminish other testosterone-dependent functions including erectile function, cardiovascular health and metabolic health.

Exposure of men to estrogenic EDCs such as bisphenol A (and structurally related "BPA replacements") has been associated with abnormal semen parameters, reduced libido and erectile/ejaculatory difficulties as well as increased risk of prostate cancer. A variety of EDCs, including certain persistent pesticides (DDT), PCBs, dioxins, flame retardants, perfluorinated compounds, and organotins) and non-persistent EDCs such as BPA and other phenols, phthalates (used as plasticizers and in some personal care products), parabens (used as preservatives in some personal care products), pesticides such as organophosphates, pyrethroids and carbamates) and solvents (e.g., glycol ethers) have been reported to have negative effects in men or test species, albeit at higher exposures than during prenatal development, and have been banned or are under regulatory consideration based on their male reproductive effects.

Can chemicals have direct (non-endocrine mediated) effects on spermatogenic cells and are such effects reversible?

Environmental contaminants may also act directly on spermatogenic cells in the testis, or maturing sperm in the epididymis, acting via a variety of cellular-molecular mechanisms. For example, fungicides that act as spindle poisons such as carbendazim, may arrest spermatogenesis at meiotic stages resulting in low sperm concentrations. NAMs that evaluate cell division or microtubule function in non-reproductive cell assays would therefore be expected to detect potential testicular toxicants which could then be tested in short term in vivo tests for testis-specific effects.

Reactive chemicals that induce oxidative stress can also damage sperm DNA. For example, components of air pollution, polyaromatic hydrocarbons, can react with DNA in late-stage spermatids or epididymal sperm forming DNA-adducts. Exposure of men to intermittently high air pollution has been associated with DNA damage in mature sperm chromatin. Such damage cannot be repaired in the male tract and may not be repairable in fertilized eggs. In such cases, embryos may fail to develop resulting in early pregnancy loss and infertility. The good news is that the damaged sperm will soon be replaced by new, undamaged sperm as spermatogenesis proceeds, provided the exposure is of short duration. Using new tiered testing schemes, in vitro tests using non-reproductive cells that detect DNA adducts, and/or reactive oxygen species can now be used to screen for potential spermatotoxicants with similar activity.

A few male reproductive toxicants are known to act only or specifically on sperm maturing in the epididymis. Alpha-chlorohydrin, derived from certain industrial processes, is one such chemical. It specifically inhibits sperm metabolism with consequent inhibition of sperm motility and resultant infertility. Such effects are typically reversible once the exposure ceases.

Other toxicants can have multiple effects on spermatogenic cells, mature spermatids or sperm in the epididymis depending upon the dose and duration of exposure. Acrylamide monomer, for example, has male reproductive toxicity in mice and rats when tested using an acute, 5-day exposure protocol. At relatively high doses, its metabolites can arrest spermatogenesis resulting in temporary infertility due to low sperm counts and with recovery after the chemical has been metabolized. At lower concentrations

that do not arrest spermatogenesis, it can alkylate sperm chromatin which may cause early embryo loss and consequently reduced litter size, effects that are reversible after the exposure is removed. Interestingly acrylamide is also a neurotoxicant and acute exposure can interfere with the motor control of breeding in rats. Based on these studies, acrylamide has been regulated to prevent hazardous exposures in the workplace or releases into the environment.

Conclusion

With increased awareness about the potential of chemicals to negatively influence many biological processes, including spermatogenesis and fertility, international research and regulatory bodies have launched a revolution in toxicity screening and testing. This revolution is developing NAMs that are more sensitive, diagnostic, and efficient while at the same time less expensive and use fewer or no animals. By pairing NAMs with what we learn about the mechanisms through which chemical exposures might affect male reproductive function, future testing can better ensure that dangerous chemicals are removed from the environment or never enter commerce to begin with. The hope is that international cooperation will continue to support new research and provide the political will needed to insure environmental health protection of male reproductive health.

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Chapter 42
Can spermatozoa be targets for drugs? If so, what are the consequences of such drug exposures?

Drugs that affect sperm structure or function; male-mediated developmental toxicity

Bernard Robaire and Barbara F. Hales

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 41). Drugs and 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. Chemicals may disturb the male germ cell genome, by mutating the DNA sequence itself, or altering the epigenome (Chapters 13-15). Finally, chemicals may be present in seminal fluid and thus have direct effects on fertilization and/or embryonic development. Effects on germ cell quality that are not repaired during spermatogenesis may decrease fertilizing ability or lead to post-fertilization adverse effects on progeny outcome. Such effects may include pregnancy loss or spontaneous abortion and birth defects, or effects manifested only later in life, such as childhood cancer, behavioral effects or learning deficits and metabolic syndrome. Paternally-mediated adverse effects on progeny may be transmitted to subsequent generations.

Male germ cells are engineered to fertilize an oocyte and to provide the 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. Adverse progeny outcomes include early or late pregnancy loss, preterm delivery or delivery of a small-for-gestational age infant, malformations, behavioral abnormalities, childhood cancer or later life onset effects, such as diabetes, breast cancer or Alzheimer disease. Animal studies

provide convincing evidence that paternal exposures to specific environmental or therapeutic agents do result in a higher incidence of adverse progeny outcomes. Treatment of male rats with cyclophosphamide, an anticancer and immunosuppressant drug, results in increased pre- and post-implantation loss as well as abnormal progeny. This drug induces sperm chromatin damage as well as epigenetic and telomere modifications, resulting in altered zygotic gene activation and a dysregulation of gene expression in the early embryo. A wide range of environmental chemicals (e.g., lead, dibromochloropropane, phthalates, bisphenols, polybrominated diphenylethers), drugs (e.g., cyclophosphamide), and lifestyle exposures (smoking, obesity) produce abnormal progeny outcomes after paternal exposure. One can deduce the stage specificity of the susceptibility of germ cells during spermatogenesis to damage based on the timing between toxicant exposure and the effect on offspring, (Chapter 9).

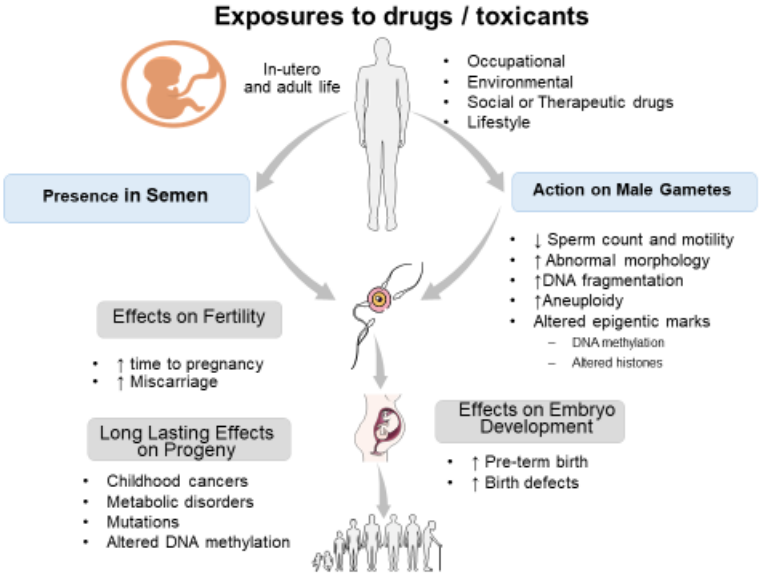

Figure 1. Paternal exposures may have an adverse impact on fertility and progeny outcome.

A number of epidemiological studies have reported 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. Therapeutic drug exposures may also be of concern with respect to progeny outcome. There is a high incidence of transient or permanent infertility after men are treated with anticancer drugs (Chapter 47). Lifestyle exposures, such as smoking, alcohol consumption and obesity, have been linked to decreased fertility and an increased incidence of some childhood cancers in progeny. The extent to which the sperm produced during recovery from chemotherapy are "normal" depends on the drug(s), doses and duration of treatment; this area of research deserves further investigation.

It is very difficult to associate a paternal exposure with a specific birth defect or childhood cancer. This is because there are relatively few studies and these are usually small, 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. Nevertheless, one should not automatically dismiss cause-and-effect relationships based on the study size. Interestingly, a clear association between drug exposure among fathers who preconceptionally took metformin for diabetes and birth defects in their male offspring, particularly genital birth defects was established in a recent large cohort study. However, other drugs, such as anxiolytics, were not found to have such effects on offspring.

It is of concern that the germ cell line of progeny may also be affected by paternal exposures, thus increasing the risk for subsequent generations (Chapter 16). 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 the DNA sequence, (Chapter 13-15). 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 the DNA methylation of paternally imprinted genes may contribute to imprinting errors and diseases in children conceived with assisted reproductive techniques (ART) (Chapter 38).

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 drugs, chemicals, and lifestyles should be made aware that there is concern with respect to an increased risk of adverse progeny outcome. Paternal counseling after exposures that are known to affect male germ cells should be highly encouraged. The development of a battery of new diagnostic tests to detect the effects of deleterious exposures on sperm chromatin and function should be a high priority.

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Chapter 43
Can we protect germ cells against testicular insults?
Male reproductive toxicology

Marvin 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. Necessary medical treatments with anticancer agents or immunosuppressive drugs, frequently cause reductions in sperm count (oligospermia); particularly treatment with radiation or alkylating drugs, can cause permanent reductions in sperm count and may even result in complete lack of sperm (azoospermia). Industrial solvents, such as bromopropane, compounds released from plastics used in food containers and medical tubing, such as phthalates and bisphenol A, and numerous fungicides and pesticides also damage spermatogenic function. Methods to prevent these damaging effects and to restore gonadal function after toxic treatments are of great importance to men who desire to have children.

Two exposure scenarios will be considered separately: (1) short-term medical treatments or accidental exposures and (2) prolonged occupational, environmental and medical exposures.

Short-term medical treatments or accidental exposures

Treatment for cancer usually involves exposures to radiation and chemotherapy during a period of several weeks to several months. Many of the anticancer agents preferentially kill proliferating cells, which in the testis are primarily the rapidly proliferating differentiating spermatogonia (Fig. 1). However, killing of differentiating spermatogonia will only result in transient damage to sperm production provided that the stem cells survive and can repopulate the tubules. Protection against these short-term effects is rarely warranted since the patients are advised against procreation at this

time because of possible genetic alterations in these developing germ cells, and the possibility that the tumor cells will also be protected.

Recovery from these short-term effects can occur as the more slowly proliferating stem cells (Fig. 1) that survive repopulate their numbers and differentiate through spermatogenesis within several months, though sometimes several years are required. But the damage might kill too many stem cells or damage the supporting cells (e.g. Sertoli, Fig. 1) or paracrine factors required for recovery. This results in prolonged azoo- or oligospermia, which are of greater consequence to the patient and protective measures would be desirable.

Accidental short-term exposure to radiation or chemicals that affect spermatogonial stem cells, such as dibromochloropropane, are also of concern. However, in these cases it is not possible to plan protective methods but only methods to stimulate recovery may be applicable.

Protection from short-term toxicant exposures

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.

Since many anti-cancer agents are selective for killing proliferating cells, the concept of placing the stem spermatogonia in a non-cycling state has been proposed as a protective mechanism. It was erroneously proposed that suppression of male reproductive hormones, [luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone] 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 dramatically enhanced the subsequent recovery of spermatogenesis by a mechanism that is still unknown. This result was not due to protection of the stem spermatogonia from being killed but rather is attributable to the restoration of the ability of the somatic cells of the testis to support the differentiation of the surviving stem spermatogonia. This strong effect seems to be unique to rats, where the stem spermatogonia are blocked from differentiating

after cytotoxic treatments unless there is a period of hormonal suppression. Hormonal suppression immediately after irradiation can also modestly stimulate the rate of recovery of spermatogenesis in mice and possibly in non-human primates. Clinical trials of protection or restimulation of spermatogenesis by hormonal suppression have generally been unsuccessful.

A more scientifically based method for manipulating stem spermatogonial cycling, would be the modulation of growth factors that are known to affect stem cell proliferation (Fig. 1), of which glial-derived neurotrophic factor (GDNF) and fibroblast growth factor (FGF) are prime examples. As yet there are no methods for reducing the proliferation of the stem cells without the loss of these cells. However, there are reports that modulation of some growth factors can stimulate the recovery of spermatogenesis in mice after exposure to busulfan, a stem cell cytotoxic chemotherapeutic drug (Fig. 1). These methods include treatment of mice with granulocyte colony-stimulating factor (G-CSF), a stimulant of hematopoietic stem cell proliferation, treatment with an inhibitor of transforming growth fact beta (TGFβ)-receptor 1, down-regulating the G-protein-coupled bile acid receptor (TGR5) signaling pathway, and injection of mesenchymal stem cells which secrete a variety of factors.

Radiation and alkylating agent chemotherapy often produce testicular damage by increasing oxidative stress by generating reactive oxygen species (ROS) and reactive alkyl groups that directly damage the DNA, lipids, and impair sperm function (Fig. 1). However, caution must be exercised about using antioxidants or radical scavengers to protect the testis against the toxic effects of ROS since protection of the tumor cells may also occur. One agent, amifostine, was originally identified because it selectively protected some normal tissues but not tumors against radiation. Although it does partially protect mouse spermatogonial stem cells against radiation, amifostine shows direct toxicity to stem spermatogonia, which in conventional fractionated anti-cancer regimens would outweigh the protective benefit. Similarly, there is some indication that it may reduce oxidative damage in the testis after chemotherapy, but there is only minimal evidence that it may enhance the survival of the spermatogonial stem cells. The benefit of this marginal survival effect is again outweighed by the fact that there appears to be increased genetic damage in the sperm produced. Most of the many other studies of antioxidant protection against radiation and chemotherapy have not addressed the effects

on stem spermatogonial cells, which is the significant target for the subsequent fertility of the cancer patient.

Anti-apoptotic compounds, such as sphingosine-1-phosphate, have been studied for protection against toxicants that kill germ cells by apoptosis (Fig. 1). This procedure partially protects differentiating spermatogonia from radiation but the question of whether stem spermatogonia were also protected was not investigated.

Reducing blood flow to testes by transient ligation or cryptorchidism has been investigated to protect spermatogenesis 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.

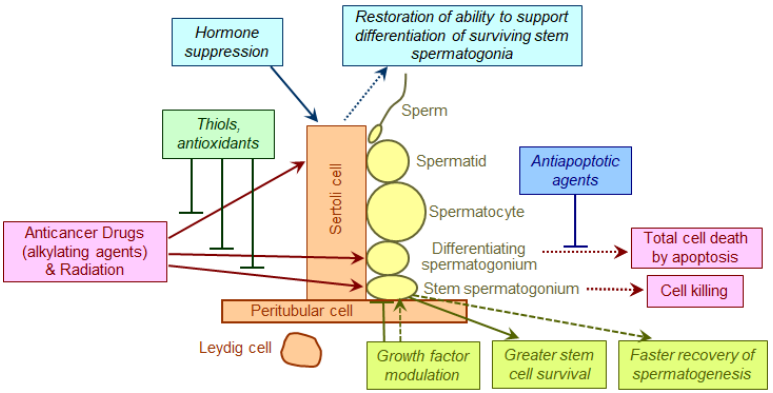

Figure 1. Diagrammatic representation of action of anti-cancer agents on specific cells of the testis and outcomes. Different targets for protection are shown: blocking the initial damage (thiols, antioxidants), restoring somatic cell function to enable surviving spermatogonia to reinitiate spermatogenesis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

Protection from Chronic Toxicant Exposures

The major emphasis in reports of attempts to protect spermatogenesis against damage from chronic exposures has been protection against oxidative stress. In addition to radiation and the alkylating agents used in cancer therapy, a variety of occupational and environmental toxicants produce testicular damage by increasing oxidative stress (Table 1). These toxicants generate ROS which are free radicals with unpaired valence electrons which are capable of oxidizing lipids, proteins, and nucleic acids. Damage occurs when these ROS overwhelm the cellular protection mechanisms, such as endogenous antioxidants like glutathione and enzymes like superoxide dismutase, catalase, and glutathione peroxidase. In addition to the process of spermatogenesis, the mature sperm function and sperm DNA integrity are also very sensitive to oxidative stress.

Table 1: Toxicants Producing Reactive Oxygen Species (ROS) and Protective Scavengers

Examples of other agents producing reproductive toxicity by oxidative stress			
Endosulfan	Hexanedione	Sodium fluoride	Acrylamide
Aflatoxin B1	Phthalates	Acetylene	Cadmium
Acetaminophen	Chlorpyrifos	Malathion	Diazinon
Silver nanoparticles	Deltamethrin	Gentamycin	Acetonitrile
Antioxidants and radical scavengers shown to protect spermatogenesis from toxicants			
Melatonin	Vitamin C	Vitamin E	Glutathione
N-acetyl-cysteine	Retinoic acid	Selenium	Doxycycline
Dietary polyphenols	Folate	Carnitines	Ubiquinone-10
Curcumin	Caffeic acid	5-acetyl sialic acid	Naringenin (flavone)
Lycium	α-lipoic acid	CeO_2 nanoparticles	Apigenin
Caryophyllene	Green tea extract	Lycopene	Royal jelly

A wide variety of antioxidants can be used to reduce tissue oxidative damage (Table 1). Most act by scavenging free radicals either directly or in concert with other scavengers. Among the antioxidants and radical scavengers are a variety of foods, fruits, vegetables, and spice products, and extracts from these products. There are numerous studies showing that these radical scavengers

can reduce damage produced by the oxidative agents to the testes of mice and rats. That is often measured by the degree of lipid peroxidation, using malondialdehyde as a marker, in the testis after exposure to ROS-producing agents; some of these studies also show that there is protection of spermatogenesis, sperm function, and/or fertility.

Despite the large literature showing protection of rodent spermatogenesis from ROS-generating toxicants, it is difficult to pinpoint a practical treatment applicable to cases of specific human exposures from environmental, occupational, or medical treatment exposures. There is debate about the benefit of consuming sufficiently high doses antioxidants to protect against oxidative damage exposure, due to concerns that consuming excessive doses of antioxidant supplements may be harmful.

There have also been numerous studies investigating the possibility that anti-oxidants can have beneficial effects in the treatment of unexplained male infertility, on the assumption that endogenous and/or exogenous ROS were contributing to the infertility. A number of these studies did show favorable effects on spermatogenesis, but others failed to do so or even showed a negative influence on the sperm parameters. This variation may be attributed to the heterogeneity of the studied population. One study with subjects who were heavy smokers of cigarettes, which produce ROS in the smoke, did show a benefit to sperm quality with daily vitamin C supplementation, suggesting protection against ROS-induced damage. However, the doses of antioxidants necessary to balance the redox system are not generally known and overconsumption of antioxidants may result in reductive stress that could cause detrimental effects on human health and well-being.

Conclusions

As yet there are no proven methods for protecting the human germinal epithelium when it is directly exposed to chemical 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 (Chapter 24) 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 grafting into a subcutaneous site, respectively, result in production of viable sperm

that yield live offspring (Chapter 8). 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 (Chapters 46, 47).

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Chapter 44
What types of testicular tumors occur in men? How effective are existing therapeutic approaches and what is the prognosis?

Ewa Rajpert-De Meyts, Lise Aksglaede and Niels E. Skakkebæk

Introduction

Testicular tumor is a commonly used general term which covers a large variety of different neoplasia that occur in the testis, some malignant, some benign. In epidemiology, testicular cancer is practically synonymous with testicular germ cell tumors (TGCT), which constitute the vast majority of all cases. TGCTs are particularly fascinating because of their origin from germ cells, which have very different biologic features than other (somatic) cells in the body. Germ cells are the only cells in the body that can undergo two different types of cell division (mitosis and meiosis), which can cause problems during transition, and they are also known for propensity to undergo apoptosis, which renders them sensitive to cytotoxic treatment. TGCT is considered a developmental disease (Chapter 45). In contrast to other solid tissue cancers, TGCT is the most common cancer occurring in young men. The peak incidence is around 25-35 years of age, which coincides with the peak reproductive activity of most men, thus fertility issues, possible hypogonadism and sexual function are of particular importance for these patients. Thanks to the advancements of modern treatment, mortality is very low, and the survival rates exceed 95% in most countries.

Interestingly, the incidence of testicular cancer is variable in world populations, with the highest rates among white men of Northern European origin (age-standardized rate of 9-11 per 100,000), and the lowest (0.5 – 1 per 100,000) in men of African and East-Asian ancestry, in whom this cancer is a rare disease. Such a big difference in the incidence indicates that the risk of testicular cancer is strongly linked to the genetic variability. Even more fascinating is the fact that the incidence of testicular cancer in each population is not stable but has been changing markedly during the second half of

the twentieth century and the beginning of the twenty first century. Some populations with historically low incidence rates, for example, Finland and Hispanic populations of the America, including in the United States, experienced a rapid increase (doubling or tripling) during recent decades. By contrast, the rates in several European countries, including Denmark and Norway, previously known for the highest incidence of testicular cancer in the world, have been showing signs of levelling off. The reasons behind these rapidly changing incidence trends are not yet known but it is obvious that environmental or lifestyle factors are involved. For further information about these aspects and possible mechanisms see Chapter 45 which deals with the origin of germ cell tumors. The focus of this chapter is on the pathology of testicular tumors and clinical management, with emphasis on andrological aspects.

Main types of testicular tumors

The testicle is a complex organ and in addition to germ cells contains different cell types that can give rise to a tumor. The tumor types that originate from testis-specific somatic cells - Leydig cells or Sertoli cells - are grouped under the name of sex-cord-stromal tumors. Other cell types, for example, epithelium of rete testis can also grow into a malignant tumor. Finally, the testis is frequently a site of hematologic malignancies, such as lymphoma (mainly in older men) or acute leukemia (mainly in young boys). All these tumor types differ significantly from each other with regard to their histology, pathogenesis, typical age at presentation, incidence, clinical course, and prognosis. This huge variety of phenotypes has been a problem for generations of pathologists and clinicians. The World Health Organization (WHO) has been updating the classification of testicular tumors regularly. In 2016 the morphology-based division was replaced by a new classification, based on the biology and cell of origin of each tumor, and the latest edition in 2022 upheld the changes. A list of main types of testicular tumors, with currently accepted names, is presented in Table 1, and those most important clinically are briefly described in the next section.

Table 1. Main types of testicular tumors.

Testicular germ cell tumors (TGCT)

• **TGCT derived from germ cell neoplasia in situ (GCNIS)**
  ○ Preinvasive: GCNIS, gonadoblastoma
  ○ Seminoma (germinoma)
  ○ Nonseminoma
  - Embryonal carcinoma
  - Yolk sac tumor
  - Choriocarcinoma and other trophoblastic tumors
  - Teratoma
• **TGCT unrelated to GCNIS**
  ○ Childhood (pediatric) tumors
  - Teratoma, prepubertal type, incl. (epi)dermoid cysts
  - Yolk sac tumor, prepubertal type
  ○ Spermatocytic tumor

Sex cord-stromal tumors
• Leydig cell tumors • Sertoli cell tumors • Granulosa cell tumors • Other or unclassified sex cord-stromal tumors
Other tumors and malignancies

• Haematolymphoid tumors
  ○ Lymphoma
  ○ Leukemia
  ○ Myeloid sarcoma
• Tumors of collecting ducts and rete testis

Testicular germ cell tumors (TGCT)

The group of testicular tumors derived from germ cells (TGCT) is the largest and clinically most important. The TGCT are divided into two main types, depending on their association (or lack of thereof) with

a preinvasive lesion, called *germ cell neoplasia in situ* (GCNIS). The commonest tumors (approximately 95%) that occur in adolescents and young adults are derived from GCNIS. Roughly half of the cases are of the type called seminoma and the other half are nonseminomas. In individuals with disorders of sex differentiation (DSD, a rare complex condition), who have poorly developed gonads, the preinvasive lesion called *gonadoblastoma* can also occur.

It is important to emphasize that the GCNIS-derived TGCTs are associated with an impaired early development of the reproductive system and belong to the so-called testicular dysgenesis syndrome (TDS), along with other abnormalities, such as cryptorchidism (undescended testis), hypospadias and infertility with testicular atrophy (small testes). Hence attending physicians must be aware of this association and always exclude a possibility of testicular malignancy in patients with these disorders. If left untreated, patients with GCNIS will eventually develop invasive tumors, although it can take several years.

GCNIS is present inside seminiferous tubules in the place normally occupied by spermatogonia (Fig. 1). In the tubules with GCNIS, Sertoli cells are always present and occasionally normal germ cells and inflammatory cells can be seen. GCNIS cells, however, are different from normal adult spermatogonia, have larger nuclei and resemble immature fetal germ cells, called gonocytes. The two cell types express embryonic markers of pluripotency and antigens normally found in primordial but not in mature adult germ cells, e.g. OCT3/4, NANOG, AP-2 gamma (TFAP2C), placental-like alkaline phosphatase (PLAP), and podoplanin (D2-40) (Fig. 1). In addition to protein-coding genes, GCNIS and all GCNIS-derived tumors, except mature teratomas, have a high expression of embryonic-type micro-RNAs (miRNA), among which the miR-371-3 cluster is clinically most important.

Seminoma is a homogeneous tumor composed of gonocyte-resembling cells that express the same markers as GCNIS cells and can contain prominent immune cell infiltrates. By contrast, nonseminomas are morphologically very heterogenous and can contain one or more of the subtypes listed in Table 1; embryonal carcinoma, yolk sac tumor, choriocarcinoma and teratoma. Because of the high expression of the above-mentioned pluripotency factors, the early tumor cells can differentiate in any type of embryonic or extra-embryonic somatic tissues.

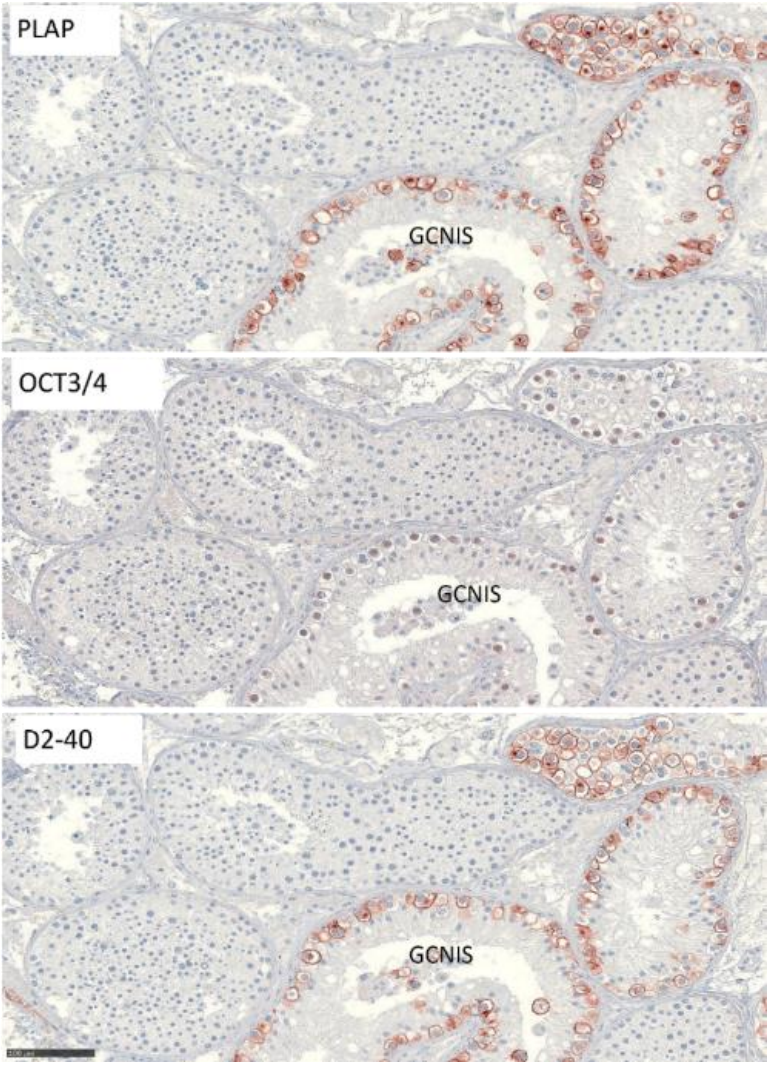

Figure 1. An example of testicular biopsy with germ cell neoplasia in situ (GCNIS). GCNIS cells are marked in several tubules by immunohistochemical (IHC) expression of PLAP (cytoplasmic), OCT3/4 (nuclear) and D2-40 (cytoplasmic). Other tubules (without IHC reaction) contain ongoing spermatogenesis, with all stages of germ cell maturation present. Scale bar equals100 μm. Reprinted from Rajpert-De Meyts et al. BJUI 2022, © The Authors.

TGCT can also occur in early childhood, typically at the age from birth to 4 years, but these tumors are rare and their incidence has not been increasing. The main types are yolk sac tumor, which has an aggressive clinical course, and mature teratoma, which is usually benign. Importantly, the childhood TGCTs are not associated with GCNIS and have different pathogeneses, which remain unknown.

A third, rare TGCT type is called *spermatocytic tumor* and occurs in relatively older men (median age at diagnosis 50-55 years). This tumor is derived from post-pubertal germ cells; spermatogonia or early spermatocytes, which can grow into a tumor as a result of genetic aberrations, with either gain-of-function mutations promoting spermatogonial proliferation or chromosomal errors blocking the progress of meiosis at the early stage and promoting polyploidy. Spermatocytic tumors have usually a benign clinical course and are treated by surgery alone.

Sex cord-stromal tumors

These tumors arise from testicular somatic cells and are much less common than germ cell tumors.

Leydig cell tumors account for approximately 3-5% of all testicular tumors, and only 1 in 10 is malignant. These tumors can occur in children and adults. The main patient groups that harbor Leydig cell tumors include boys with peripheral precocious puberty, men with unexplained infertility, gynecomastia or Klinefelter syndrome. Among genetic defects, activating mutations of the luteinizing hormone (LH) receptor have been best described. Leydig cell tumors are usually small, well demarcated and are detected by ultrasound examination.

Sertoli cell tumors are rare and can occur in children and adults, sometimes in patients with undescended testes and in men with Klinefelter syndrome. Two subtypes of these tumors, both with prominent calcifications, occur in association with Carney complex and Peutz-Jeghers syndrome; these are multiple neoplasia syndromes with a dominant inheritance characterized by non-endocrine and endocrine tumors, adrenocortical disease, skin hyperpigmentation and gynecomastia.

Diagnosis of testicular tumors

Diagnosis at the precursor stage of GCNIS is rare because this lesion is asymptomatic. GCNIS is usually an incidental finding in patients

from risk groups (see next section *Prevention*). Overt testicular tumors are often reported by the patients themselves, if large enough to be felt during self-examinations, or are found during andrological work-up that includes physical examination and scrotal imaging, usually by ultrasound. If a malignancy is suspected, blood tests must follow to measure biochemical markers secreted by germ cell tumors. Serum tumor markers include human chorionic gonadotropin (β-hCG) secreted by trophoblastic cells and choriocarcinoma, alpha fetoprotein (AFP) secreted by yolk sac tumors, and lactate dehydrogenase (LDH) produced by seminoma. These markers are raised in only 60% of patients with TGCTs. Recently, a new blood test has been developed – it is based on the detection of GCT-specific micro-RNAs (miR-371a-3p cluster). This test is sensitive and helps to diagnose practically all TGCTs, except mature teratomas. There are currently no blood tests to detect the sex cord-stromal tumors, except Leydig cell tumors, which usually secrete excessive amounts of steroid hormones, and can be manifested by peripheral precocious puberty in boys or gynecomastia in any age.

The final diagnosis of testicular tumors, including preinvasive lesions, must be confirmed by the histopathologic analysis of the excised tissue. Here, a panel of clinically useful immunohisto-chemical markers is very helpful. The similarity of GCNIS and seminoma to gonocytes is exploited by several markers not otherwise present in the adult testicle (Fig. 1). Recognition of different components of nonseminomas is essential, because these tumors have worse prognosis than pure seminoma. The immunohistochemical staining for the presence of AFP, HCG, or SOX2 can be helpful to reach correct diagnosis.

Prevention

Because of unknown etiology it is not yet possible to prevent testicular cancer in the general population, but the invasive cancer can be prevented in patients from high-risk groups by early diagnosis at the GCNIS stage. The main risk groups include DSD, history of cryptorchidism, poor semen quality or infertility, especially if there are signs of testicular dysgenesis, such as small testicular volume or microlithiasis (microcalcifications on ultrasound). GCNIS is present in the other testicle of about 5% of patients with unilateral TGCT, and its detection can significantly reduce the risk of metachronous bilateral cancer.

Currently, the diagnosis of GCNIS requires testicular biopsy, which in at-risk patients should be bilateral, while in patients with TGCT a contralateral testis biopsy can be performed at the time of orchiectomy for the primary tumor. The biopsies should be large enough and evaluated carefully, with obligatory immunohistochemical staining for at least one GCNIS marker to avoid overlooking the neoplastic cells (Fig. 1). Ongoing research efforts aim to develop a detection method less invasive than biopsy. GCNIS cells can be detected in semen by an immuno-cytological method, and the above-mentioned miRNA test in blood can detect about half of patients with GCNIS but further improvement of sensitivity is needed for routine use of these approaches.

Management, prognosis and late effects

Radical removal of the testis (orchiectomy) is the treatment of choice for malignant testicular cancer, including the vast majority of the TGCTs. Other tumors, for example small and well-demarcated Leydig cell tumors, which are often benign, can be treated more conservatively by testis-preserving surgery, when the surgeon carefully removes only the tumor tissue. In all young adults with testicular cancer, semen cryopreservation ought to be offered before surgery or any other treatment.

Patients with GCNIS alone can be treated by orchiectomy of the involved testicle or by low-dose radiotherapy in patients with bilateral GCNIS, which can eradicate the malignant cells while preserving Leydig cells and androgen production. All patients with malignant TGCT must be evaluated for a possible spread of malignancy, which would define their prognosis and treatment. Prognostic staging takes into account the levels of the above-mentioned circulating serum tumor markers, primary tumor type and size, and the presence of distant metastases. The patients are subsequently stratified to the good, intermediate, or poor prognosis groups.

The rates of survival in patients with testicular cancer are in general excellent, mainly because of good methods of diagnosis, surgical treatment, and the very efficient chemotherapy regimens based on cisplatin in combination with other cytotoxic drugs. For the US population (2008-2014), the 5-year relative survival rate was overall >95%, but as high as >99% if the tumor was detected in early localized stage. For disseminated testicular cancer, the survival rate drops to 70-75%, underlying the importance of early diagnosis. The greatest management challenges are in patients, who do not

respond to therapy and have recurrent tumors. The patients with poor prognosis (survival rates 50-60%) are treated in experienced oncology centers, with aggressive salvage regimens containing additional cytotoxic drugs. Patients treated with radio- or chemotherapy are at an increased risk of ototoxicity and hepatoxicity, peripheral neuropathy, cardiovascular disease, and second cancers.

Most testicular cancer survivors are young men and have several decades of life after treatment. Even though most of them will be declared 'cancer-free', they should be followed for many years, not only with regard to the possibility of a late relapse, but also the health issues caused by loss of one or both testes, which can contribute to numerous health problems and reduced life expectancy. The most common problems include infertility and testosterone deficiency, which in some patients occur even before the appearance of cancer, due to the frequent association of TGCT with a general impairment of testicular function. Other possible health problems are sexual dysfunction, psychological stress, metabolic syndrome, and osteoporosis later in life. Each patient ought to be cared for by a team of collaborating specialists, including an andrologist.

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Chapter 45
What do we know about the origin of germ cell tumours?

Kate L. Loveland and Daniela Fietz

Why is it important to understand this?

Testicular germ cell tumours are the most commonly diagnosed solid tumour in 15-44 year old men, ranging in incidence from 1:1000 to 1:100,000, depending on location. In Chapter 44, the clinical importance of testicular germ cell tumours (TGCTs) to the reproductive health of young men was presented. There is a good prognosis with a >95% 5-year survival rate in men being diagnosed at an early stage in the US. Upon improvement of non-invasive diagnostic tools, early diagnosis will become easier. However, if the tumour has spread or is refractory to therapy, the picture worsens significantly to 75% survival or below. The added burden of co-morbidities and associated risk of infertility linked with a TGCT diagnosis are of concern to patients, their families and health care providers. The risk of poor outcomes for men with this disease will be increased in settings of limited medical consultation or in the absence of encouragement to seek an early diagnosis when a testicular lump is detected.

In addition to the potential of genetic risk factors to impair adult male reproductive health and testis function, accumulating evidence shows that early life exposures, particularly those *in utero*, have a substantial impact. TGCT incidence is increasing in many parts of the globe, particularly where environmental stressors (e.g. industrialisation, lifestyle factors) and genetic risks are at play. Rates of increase of 2% per annum and higher are reported for many countries. In Australia, the age-standardized rate increased from 4.2 cases to 7.5 per 100,000 males between 1982 and 2017; in several European countries, including Germany, an increase of nearly 3% per annum is predicted, while those with the highest rates (e.g. Denmark) are expected to experience a decline. These numbers highlight the need to learn what contributes to TGCT risk that cannot be attributed to genetic factors. Alongside the higher TGCT incidence is the escalating prevalence of associated testicular dysgenesis

pathologies, including male infertility, hypospadias and cryptorchidism. The accumulating evidence that tumours within other organs arise from male germline cells provides additional clinical relevance to understanding the genesis and outcomes of inappropriate germ cell development.

Clinical and biomedical research in Andrology that builds knowledge of TGCT origins will be relevant to developing approaches to limit both its incidence and spread. The long-standing goal is to provide evidence of how to reduce TGCT incidence and testicular dysgenesis through changes in lifestyle, manufacturing and medical practices, and ultimately influence public policy.

The fundamental information

Although direct observations are lacking, human germline cells are considered to be specified during the second week of gestation (GW2) as primordial germ cell (PGC) precursors, under the influence of bone morphogenetic proteins (BMPs), Activins and WNT signalling proteins. Moving to the extraembryonic endoderm, they appear as large rounded TNAP (tissue non-specific alkaline phosphatase)-positive cells with a large nucleus and clear cytoplasm. This physical separation of PGCs from somatic cells is required for their unique developmental trajectory; near-complete erasure and replacement of genomic epigenetic marks involving DNA methylation is essential to form gametes that can produce healthy offspring (Chapter 13). The sexually indifferent PGCs migrate to the gonadal ridge by ~GW5. In nascent testes, somatic cells expression of *SRY* establishes male fate by GW8. Now termed gonocytes or pre-spermatogonia, germ cells become enclosed into cords by Sertoli cells, in an epithelium that will surround and support spermatogenesis throughout life. In rodents, gonocytes proliferate, then enter a key phase of quiescence and epigenetic reprogramming (re-methylation) spanning one week; after birth they resume proliferation, migrate to the cord perimeter, and differentiate into spermatogonia. In contrast, human gonocytes do not develop as a single, synchronous population. By ~ GW13, some move from the cord centre to perimeter, displaying decreased OCT4 and increased NANOS2 and DDX4 levels. This transformation of gonocytes into prespermatogonia proceeds through the 2nd trimester and is accompanied by cell cycle exit, with this transformation complete by the hormonal surge of mini-puberty occurring by 4-6 months postpartum.

As the embryo develops, the various testicular somatic cells multiply and differentiate, and immune cells enter the testis. An important period of embryonic development involves the synthesis of testosterone (T) at levels that drive masculinization. Fetal testis steroid production involves both Leydig cells that convert cholesterol to androstenedione (A4) and Sertoli cells that convert A4 to T. The key window of masculinization in human embryos (~GW8-14) marks the period when disruptions to normal steroid production can derail the normal developmental trajectories of the testis, the male reproductive tract (e.g. reduced anogenital distance), and potentially influence other organs.

Understanding TGCT origins

Successive observations by clinician-scientist Niels Erik Skakkebaek yielded the first evidence that TGCTs develop from fetal germline cells that persist in the adult testis. He documented histological observations from two men presenting with infertility. The initial biopsy for each showed abnormal tubules containing only Sertoli cells and mitotically active, possibly tetraploid, cells appearing similar to spermatogonia but with enlarged nuclei. Subsequent biopsies revealed embryonal carcinoma (EC) detected 16 months later in one man, and atypical germline-like cells 4.5 years later in the other. These findings revealed that the cells we now term germ cell neoplasia *in situ* (GCNIS; formerly named carcinoma *in situ*, or CIS cells) progress to form the two main TGCT subtypes: non-seminoma (EC-like) and seminoma (similar to gonocytes).

Subsequent decades of histological and molecular analyses provided strong evidence that TGCTs arise from fetal germline cells that do not differentiate properly and are not eliminated by apoptosis. Robust markers of germline differentiation status illustrate that the expected trajectory of progressive transcription factor expression does not take place in these cells. Many markers found in human primordial germ cells and gonocytes are present in GCNIS cells, and persist in seminomas, reflecting the gonocyte-like phenotype of these tumours and the repression of differentiation. These include *PRDM1*, previously named *BLIMP1*, a hallmark transcriptional repressor protein in germline cells, in addition to *POU5F1* (encodes OCT4), *LIN28*, and *NANOG*. *SOX17* is present in PGCs, gonocytes and seminomas, but absent from non-seminomas which in contrast express *SOX2*, a direct target of PRDM1, as an indicator of their more differentiated status; both of these can

heterodimerize with OCT4 to maintain pluripotency. Non-seminomas also express pluripotency markers, *POU5F1, LIN28*, and *NANOG*, in addition to *GDF3, TDGF1* (encodes CRIPTO), and *DNMT3B*, a profile which aligns them to embryonic stem cells.

Genome-wide association studies (GWAS) have identified many candidate mutations for TGCTs, but no driver mutation for all subtypes, highlighting the expectation that environmental factors affecting *in utero* development provide the key to understanding the risk of GCNIS cell formation that underpins the growing incidence of TGCT. GCNIS and their derivative TGCT cells are typically aneuploid. The most common genetic rearrangement in GCNIS cells that progress to TGCTs is isochromosome 12p gain; mutations in *KIT* (encodes a signalling receptor), *KRAS* and *NRAS* (encode signal transduction proteins) and CpG island undermethylation are hallmarks of seminomas, while hypermethylation (associated with gene silencing) of genes associated with DNA repair, is documented in non-seminomas. These differences in methylation indicate that there are several mechanisms or routes by which these cells become vulnerable to disruption.

Disruptions to masculinization programming

Disruption of steroid hormone activity in the masculinization programming window (MPW) affects the male germline; the timing of vulnerability in rodents (rat, embryonic day [E]15.5-18.5, mouse, E13.5-E15.5) coincides with the lowest genomic methylation levels and when gonocytes become (and are) quiescent. The human MPW is considered to occur from GW8-14 in fetal human testes.

Steroid disruptions in the MPW affect somatic cell functions, resulting in focal areas with fetal Leydig cell aggregations and Sertoli cells outside cords; such changes could influence the germline indirectly. In rodent *in vivo* and *in vitro* studies, germ cells exposed to phthalates can appear as multinucleated and also outside of the cords. However, evidence from studies of mice lacking normal androgen receptor function demonstrate that germ cells may be directly affected in the absence of appropriate stimulation, with some delay in entering quiescence. Altered levels of testosterone as well as estrogens can reduce germline differentiation and survival during this crucial window.

Formation of the male phenotype requires differentiation of both internal and external genitalia. In rodents, this is stimulated by fetal testis-derived androgens,;however in humans, placental-derived hCG

and androsterone are synthesised in the placenta, and fetal liver and adrenal gland also produce androgens. Testes from rodents exposed to endocrine disrupting environmental chemicals, such as dibutyl phthalate (DBP), exhibit changes that simulate phenotypes seen in humans, including reduced anogenital distance in males, cryptorchidism, hypospadias, and reduced fertility. This spectrum of phenotypes, collectively termed Testicular Dysgenesis Syndrome (TDS) (Chapter 44), may be mechanistically linked due to their co-association, including with increased risk of TGCT formation. For example, cryptorchidism is linked with a 4-fold greater risk of testicular cancer.

What pathways, cells, and factors contribute to GCNIS?

How GCNIS cells emerge and persist in the fetal human testis cannot be directly determined for ethical and practical reasons, and no experimental animal models reliably recapitulate this pathology. A highly regarded concept is that two stages of germline changes are required to generate TGCTs, and the extended period of human germline quiescence (spanning 1 or more years) is permissive, in contrast to the week interval of germline quiescence in rodent models. Cultures of fetal human testes have been used to validate hypotheses regarding what signalling pathways are involved when the somatic cell environment facilitates survival and/or proliferation of neoplastic germ cells. Fragments (1^{st} and 2^{nd} trimester) exposed to the signalling protein NODAL contained greater numbers of OCT4+ cells, although this pathway activity and this marker is normally downregulated as gonocytes transition into pre-spermatogonia. The NODAL co-receptor, CRIPTO, is a biomarker for TGCTs that can be detected in the serum of men with GCNIS cells, correlating positively with a second biomarker, miR-371a-3p.

The future

Simultaneous interrogation of multiple markers may eventually indicate tumour cell identity and status, to guide appropriate interventions. Roles and responses of other somatic cells, especially immune cells, are under investigation for their roles in fetal testis growth and TGCT aetiology. This fundamental research should one day explain why some immature germ cells are inappropriately

retained in the adult testis, and what governs their transition into neoplastic cells.

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Chapter 46
Can we preserve testicular germ cells of prepubertal patients for later fertility?
Next generation therapies to preserve fertility and treat infertility

Sarah K. Munyoki and Kyle E. Orwig

Chemotherapy and radiation treatments for cancer or other conditions can cause infertility. Similar treatments used for myeloablative conditioning prior to bone marrow transplantation for non-malignant conditions (e.g., sickle cell disease, β-thalassemia) also can cause infertility. Another potential cause of infertility is gender affirming treatments for gender dysphoria. Adult survivors of childhood cancers and transgender or gender non-conforming teenagers express a desire to have biologically related children. Therefore, all patients should be educated about the impact of their diseases or medical treatments on future fertility and about options to preserve fertility. Adolescent and adult people with testes have the option to cryopreserve a semen sample with sperm prior to gonadotoxic treatment; sperm can be thawed in the future and used to achieve pregnancy using standard assisted reproductive technologies such as in vitro fertilization (IVF) (Chapters 24, 35). That option is not available to prepubertal patients who are not yet producing sperm or for those with gender dysphoria who do not want to discontinue their gender affirming treatments to produce sperm. The only fertility preservation option for those patients is testicular tissue cryopreservation (TTC) (Chapter 12). We will review several techniques that are currently in the research pipeline and may be available in the future to mature frozen/thawed testicular tissues and produce sperm that can be used to alleviate infertility.

Spermatogonial Stem Cells and Spermatogenesis

Spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis, the process that produces sperm in the testes

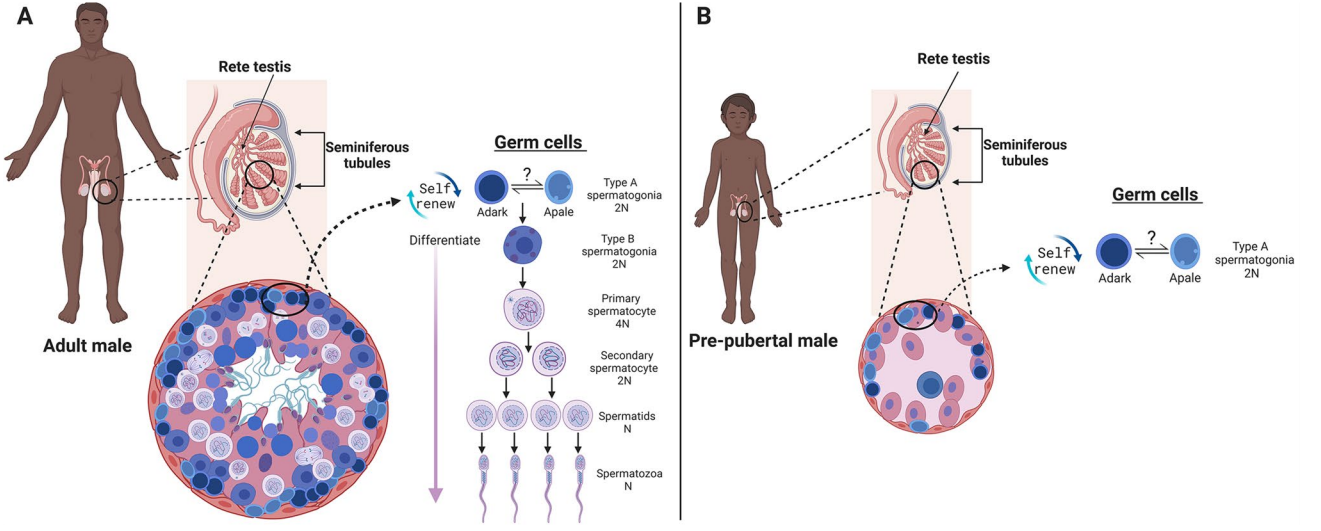

Figure 1. Stem cells, spermatogenic lineage development and testis anatomy in adult and prepubertal males. A) Spermatogenesis occurs in the seminiferous tubules of the testis. The spermatogonial stem cells (SSCs) that are at the foundation of spermatogenesis are thought to be among the pool of A-dark and A-pale spermatogonia. **B)** Prepubertal boys do not have spermatogenesis in their seminiferous tubules, but they do have A-dark and A-pale spermatogonia that have the potential to produce sperm. Created with BioRender.com.

(Chapter 8). SSCs or their precursor prospermatogonia (gonocytes) are present in the testis before birth. At the time of puberty, under the influence of gonadotropic hormones from the pituitary, SSCs initiate spermatogenesis and maintain continuous sperm production throughout postpubertal life. This is possible because SSCs precisely balance self-renewing divisions that maintain the stem cell pool with differentiating divisions that give rise to spermatogenesis. In humans, SSCs are described as A-dark and A-pale spermatogonia based on the intensity of their nuclear staining with hematoxylin. A-dark and A-pale spermatogonia are located on the basement membrane of seminiferous tubules and may undergo one or two transit amplifying mitotic divisions before giving rise to differentiating type B spermatogonia, that divide one more time to produce primary spermatocytes. Two meiotic divisions give rise sequentially to secondary spermatocytes and then haploid spermatids, which undergo spermiogenesis to produce terminally differentiated sperm (Fig. 1A) (Chapter 9). Chemotherapy and radiation treatments are toxic to the spermatogenic germ cells in the testis and can cause temporary or permanent infertility. Depending on the treatment, up to 70 percent of cancer patients will recover spermatogenesis after treatment because at least a portion of the stem cell pool survived treatment and could regenerate spermatogenesis. The speed of spermatogenic recovery is likely related to the size of the surviving pool of SSCs. The remaining 30 percent of cancer patients are rendered permanently infertile by their treatment, and this is likely caused by complete depletion of the stem cell pool and/or damage to testicular somatic cells that are necessary to support spermatogenesis.

Testicular Tissue Cryopreservation

Prepubertal boys are not producing sperm, but they do have A-dark and A-pale spermatogonia in their testes that have the potential to produce sperm (Fig. 1B). Clinics around the world are actively cryopreserving testicular tissues from young patients in anticipation that those tissues can be thawed in the future and matured to produce sperm. Immature testicular tissues are typically collected by wedge biopsy of one or both testes and tissues have been cryopreserved for patients ranging in age from a few months old to >18 years old. All reporting centers use slow freezing and most use DMSO as the cryoprotectant (Fig. 2A). Over 2,500 testicular tissues have already been frozen for patients worldwide (unpublished,

informal discussions among a network of international providers) and many are already approaching their reproductive years. It is necessary to develop next generation reproductive technologies that will allow patient-survivors to use their tissues for reproduction, and responsibly translate them into clinical practice. There are several testicular cell-based and tissue-based technologies in the research pipeline that are summarized in Fig. 2.

Using Cryopreserved Testicular Tissues to Produce Sperm

Autologous Spermatogonial stem cell transplantation. SSC transplantation was first described in mice by Brinster and colleagues in 1994 and has now been translated to numerous animal models. Transplanted stem cells regenerate spermatogenesis in the seminiferous tubules of the testis with the production of fertilization competent sperm that have produced embryos or offspring in mice, rats, goats, sheep and monkeys. Under some circumstances, transplanted stem cells can restore natural fertility. However, small biopsies obtained from prepubertal boys contain only a small number of SSCs, which are likely to regenerate small pockets of spermatogenesis in the testis but not restore natural fertility. Nonetheless, testicular sperm can be retrieved surgically and used to fertilize oocytes by IVF with intracytoplasmic sperm injection (ICSI, Fig. 2B). Testicular sperm do not swim, so cannot fertilize by intrauterine insemination (IUI) or conventional IVF. Methods to expand spermatogonial stem cell numbers in culture are well-established in rodents, producing stem cell numbers sufficient to restore natural fertility. Methods to maintain and expand SSCs in culture have not been translated to any higher animal models or humans, although this is a very active area of research.

Autologous testicular tissue grafting. Testicular tissue grafting involves placing intact pieces of immature testicular tissue under the skin where it matures to produce fertilization competent sperm and offspring. In this approach, SSCs are maintained in their cognate seminiferous tubule niches in intact pieces of testicular tissue. The goal of testicular tissue grafting is not to restore natural fertility since the grafted tissue is not connected to the excurrent duct system. The goal is to mature grafted tissue to produce sperm that can be recovered for fertilization by ICSI to produce offspring

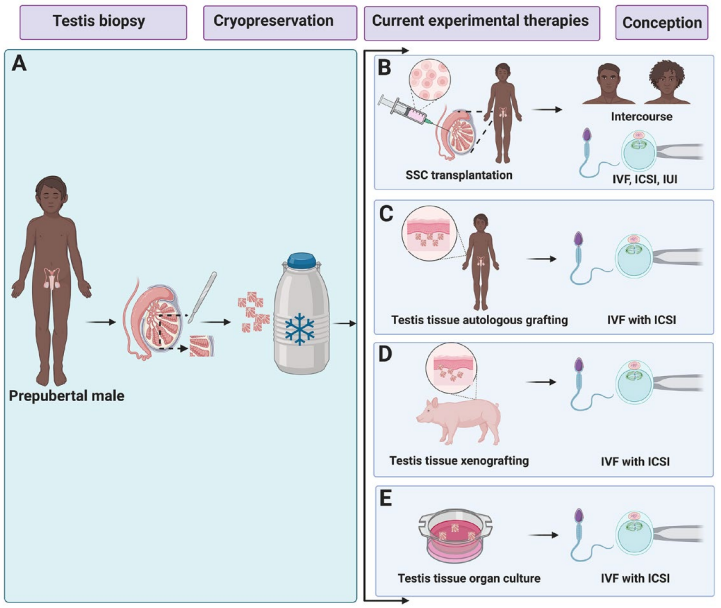

Figure 2. Fertility preservation and fertility restoration for people with testes. A) Testicular tissue cryopreservation is the only fertility preservation option available to prepubertal boys who are not making sperm. Testicular tissues are cut into small pieces (2-5 mm diameter) and cryopreserved for future use. Freezing intact pieces of testicular tissue preserves the option for cell- or tissue-based therapies in the future. **B)** Spermatogonial stem cell (SSC) transplantation. Frozen and thawed testicular tissues are digested with enzymes to produce a suspension of cells that is injected back into the seminiferous tubules of the testis. This method can lead to regeneration of spermatogenesis in the testis and possibly restore fertility. If sperm counts are low, sperm can be used to achieve pregnancy using standard of care assisted reproductive technologies (IVF, ICSI, IUI). If there are no sperm in the ejaculate, regenerated sperm can be retrieved surgically from the testis and used to fertilize by IVF with ICSI. **C)** Testicular tissue grafting. Frozen and thawed testicular tissues are grafted under skin of the patient where they can mature to produce sperm. Graft-derived sperm can used to fertilize by IVF with ICSI. **D)** Testicular tissue xenografting. Frozen and thawed testicular tissues are grafted under the skin of an animal host where they can be matured to produce sperm. Graft-derived sperm can be used to fertilize by IVF with ICSI. **E)** Testis tissue organ culture. Frozen and thawed testicular tissues are placed in tissue culture and matured to produce sperm that can be used to fertilize by IVF with ICSI. Created with BioRender.com

(Fig. 2C). Homologous species testicular tissue grafting was first reported in mice by Dobrinski, Schlatt and colleagues and was more recently translated to nonhuman primates with the production of fertilization-competent sperm and a baby monkey.

SSC transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous mammalian species with production of fertilization competent sperm and live offspring. These technologies may be ready for translation to the human clinic for patients who have cryopreserved their testicular tissues or cells. The autologous transplantation approaches described above may not be appropriate for all patients. It may not be safe to transplant testicular tissues or cells from leukemia patients or patients with testicular cancer due to the potential risk of reintroducing malignant cells to the survivor. Transgender subjects may not want to experience the male puberty with testosterone production that would be required to mature their testicular cells or tissues inside their own bodies. Therefore, outside-the-body approaches are needed to mature the cryopreserved immature testicular tissues to allow production of sperm for reproduction.

Testicular tissue xenografting. A potential alternative to autologous testicular tissue grafting is to graft tissues into an animal host (Fig. 2D). Immature testicular tissues from pigs, goats, rabbits, hamsters, dogs, cats, horses, cattle and monkeys have been grafted under the back skin of immune-deficient nude mice and matured to produce spermatids or sperm. This approach is effective with immature or prepubertal tissues but not with adult tissues, so it may be an ideal option for prepubertal patients who have cryopreserved testicular tissues. Xenograft-derived sperm have been used to fertilize and produce offspring in rabbits, pigs and monkeys. Prepubertal human testicular tissues survive in mouse hosts and initiate spermatogenesis up to the spermatocyte stage, but production of sperm from those tissues has not been reported. Future directions may include treating mouse hosts with hormones or growth factors to promote complete spermatogenesis from xenografted human tissues or to test alternative animal hosts. Using xenograft-derived sperm in reproduction may raise concerns about xenobiotics that could be harmful to human health, but the path forward is already being paved by initial reports transplanting pig organs into human patients.

Testicular tissue organ culture. Another alternative to autologous transplantation approaches is to mature prepubertal testicular tissues in vitro (Fig. 2E). Ogawa and colleagues performed ground-breaking work on the method of testicular tissue organ

culture at the air-gas interface in mice. Briefly, small pieces of testicular tissue from neonatal mice were placed on an island of agar that was half soaked in tissue culture medium. Tissue was in direct contact with the air on the upper surface and in direct contact with media-soaked agar on the bottom surface. Spermatids or sperm collected from the tissues between 20-42 days were competent to fertilize and produce offspring. Supplementing culture medium with knockout serum replacement (KSR) instead of or in addition to fetal bovine serum (FBS) was critical to achieving complete spermatogenesis. The production of haploid spermatids or sperm started to decline after 30-40 days in culture but persisted up to two months. Also, the tissues mounded up on top of the agar island. Complete spermatogenesis was observed only in tubules on the outer surface exposed to the air interface while tubules in the center of the mound were empty or necrotic. The same group later developed different variations of microfluidics devices to maintain testicular tissues in a monolayer of seminiferous tubules for 4-6 months with continuous production of haploid cells. If this approach can be replicated by other groups in mice and translated to higher animal models, including humans, it may have important implications for cancer patients or transgender subjects where it would be unsafe or inappropriate to transplant cryopreserved testicular tissues or cells back into their bodies.

Concluding Thoughts and Future Directions

In this chapter, we reviewed next generation technologies that may allow patients who cryopreserved their immature testicular tissues when they were young to use those tissues to produce sperm and have children. The autologous methods of spermatogonial stem cell transplantation and testicular tissue grafting are mature technologies that have been replicated in several animal models and may be ready for translation to the clinic. When those methods are deployed in the clinic, it is important to manage expectations of the patient and of the medical/research community. Experiences with ovarian tissue cryopreservation and ovarian tissue transplantation in human patients may be instructive. The first reports of ovarian tissue cryopreservation were in 1994 and the first reports of autologous re-transplantation of those tissues with return of hormone production, menstrual cyclicity and offspring were in 2004 and 2005. There are now over 130 reported live births from transplanted ovarian tissues. Those outcomes led the American Society for Reproductive Medicine

to advise that the experimental label could be removed from ovarian tissue freezing, which may help reduce barriers in access to care. However, it is important to note that it was not always possible to determine whether pregnancies were from transplanted tissues or surviving endogenous follicles; most of those live birth outcomes were from women who were already adults when they cryopreserved their tissues, many of whom returned to used their tissues within a decade of their cryopreservation. There is, thus far, only one documented live birth from ovarian tissue that was prepubertal at the time of cryopreservation.

To our knowledge, the first reports of testicular tissue cryopreservation for prepubertal boys were in 2005 and there are no reports of transplanting those testicular tissues or cells back into patients. As indicated above, in the absence of methods to expand SSC numbers in culture, SSC transplantation is likely to regenerate focal areas of spermatogenesis in the testis but unlikely to restore natural fertility. Also, because these are autologous transplants back into the seminiferous tubules of the testis, it will not be possible to know whether sperm are from transplanted SSCs or from endogenous SSCs that survived the gonadotoxic treatment. Finally, since most patients were children at the time that they cryopreserved testicular tissues, it may be many years before the first patients attempt to use their tissues for family building. Will it be necessary to document 130 live births to remove the experimental label from testicular tissue cryopreservation? Perhaps it would be adequate to show that transplanted testicular tissues can be matured to produce "normal" sperm. Since grafted tissues will be placed under the skin and separate from the endogenous testis, the origin of sperm derived from those grafts will be unequivocal. If graft development occurs in a time course similar to what was observed in nonhuman primates, sperm outcomes could be known within a year of transplantation. Graft derived sperm can be used immediately or cryopreserved for the patient's future use, providing assurance that their future fertility using standard assisted reproductive technologies is secured. For the medical/research community, this will provide evidence of the reproductive potential of cryopreserved, prepubertal testicular tissues that may justify removing the experimental label from testicular tissue cryopreservation.

Autologous transplantation will not be appropriate or safe for all patients. We reviewed testicular tissue xenografting and testicular tissue organ culture as methods to mature prepubertal testicular tissues and produce sperm outside the body. More work is needed to

demonstrate that those methods are robust, reproducible across species and safe before they can be translated to the human clinic. Other methods that are in the research pipeline but were not described in this chapter including de novo testicular morphogenesis, in vitro gametogenesis from testicular cell organoids and in vitro gametogenesis from patient-derived pluripotent stem cells. Those methods are early stage but may expand options to remove malignant cells before transplanting patient testicular cells back into their bodies or for producing sperm outside the body. Assisted reproductive technologies that produced the world's first "test tube" baby, Louise Brown (born July 25, 1978), have now produce over 6 million babies worldwide. Louise was possible because her dad was able to produce sperm and her mom was able to produce eggs that were combined in the laboratory to achieve in vitro fertilization by the team of Patrick Steptoe (Physician) Bob Edwards (Researcher, Nobel Prize winner) and Jean Purdy (Research nurse). We may be at the dawn of a new era in reproductive medicine where it will be possible to help patients who are not able to produce mature eggs or mature sperm to have biologically related children.

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Chapter 47
What is the impact of cytotoxic and surgical therapy on sperm and fertility preservation in cancer patients?

Corey A. Faw and Robert E. Brannigan

Approximately half of men will receive a cancer diagnosis in their lifetime. Due to advancements in therapeutic options, a majority of patients will survive their cancer, with 5-year survival rates of 76% for males up to 40 years of age and 85% for males up to 19 years of age. These outstanding survival outcomes have led to a greater emphasis on patient survivorship issues in the wake of cancer treatment, which can cause irreversible damage to spermatogenesis and male fertility potential. These considerations, along with the increasing frequency of men pursuing biological parenthood later in life, have collectively led to the emergence of fertility preservation as an essential consideration when planning oncologic therapy.

Even prior to the initiation of cancer treatment, male fertility is often impaired. Authors from the CECOS network in France have shown that males with testicular cancer as well as those with lymphoma have lower semen parameters at the time of cancer diagnosis, including lower sperm concentration, sperm motility, and total motile sperm count compared to fertile controls. Williams et al. reported that prior to treatment, 52% of males with testicular cancer and up to 30% of males with other types of cancer were oligospermic upon presentation. Spermatogenesis and normal fertility requires an intact hypothalamic-pituitary-gonadal (HPG) axis, which can be disrupted through endocrine, inflammatory, and other immune responses to malignancy. This subfertility at baseline can be worsened by systemic processes, including malnutrition and fever, that oncologic patients often endure.

Oncologic Therapies Overview

While their mode of action varies, both chemotherapy and radiation induce DNA damage or slow replication, preferentially targeting rapidly dividing cell lines. Within the testes A (pale) spermatogonia are the progenitors of spermatogenesis and under constant turnover (Chapter 9). A (dark) cells are more quiescent, serving as a reserve pool during times of significant stress or toxicity. This process allows a natural resilience to testicular insults, but with sufficient dosages, both lines can be depleted leading to permanent infertility. In contrast to spermatogonia, Leydig cells have a low rate of division leading to their resilience to such therapies. Though cytotoxic agents can ultimately cause hypogonadism, the relative sensitivities of these cell types result in a disproportionate effect on fertility over testosterone deficiency. For decades the notion of fertility preservation was unattended to in the course of cancer therapies, leaving many patients cured of their cancer but permanently infertile as a side effect. An abundance of literature shows that most patients of reproductive age will survive their cancer therapies and desire the option to pursue biological paternity at some point after treatment. Those patients who have lost their fertility potential in the wake of cancer treatment and who did not undergo sperm cryopreservation report high levels of regret and anxiety related to this outcome. Therefore, it is imperative to understand the impacts of cytotoxic therapy on fertility to appropriately manage patient care and survivorship.

Radiation Therapy

Radiation is a cornerstone of cancer treatment. Worsening reproductive outcomes are associated with fractionated therapy (vs. non-fractionated therapy), increasing radiation dosage, increasing patient age, and the concurrent administration of other therapies. Patients at highest risk are those receiving direct testicular radiation or whole-body radiation. Interestingly, the Leydig cells are typically spared functionally until up to 20-30 Gy of radiation has been administered. However, the lineages of cells involved in spermatogenesis are much more radiosensitive, with testicular radiation dosages < 0.8 Gy commonly causing transient oligozoospermia, 0.9 -2.0 Gy typically resulting in transient azoospermia, and dosages ≥ 2.0 Gy commonly resulting in permanent and irreversible azoospermia. The nadir in sperm production for a given patient typically occurs 3-6 months after the initiation of radiation therapy, and recovery usually occurs over 1-

3 years, with lower testicular radiation dosages being associated with faster recovery of spermatogenesis.

Radiotherapy (XRT) is employed for a variety of solid tumor and hematologic malignancies in addition to preparation for bone marrow transplantation. Direct exposure to the testes, scatter from nearby radiation, and disruption of the hypothalamic-pituitary-gonadal axis through cranial radiation can all impair fertility.

Cumulative XRT is not the only determinant of gonadotoxicity; the manner in which radiation is delivered is also important. Fractionated dosing has a lower threshold for injury. It is hypothesized that the chronic damage from lower doses of XRT is insufficiently repaired, allowing more mutations to accumulate, leading to enhanced apoptosis. In addition to the radiation therapy schedule, the rate of delivery also can impact fertility. One study paradoxically showed low dose rates of radiation lead to more testicular damage compared to high dose rates. Likewise, a recent study showed that ultra-high dose rate (FLASH) radiotherapy (>40 Gy/s) demonstrated remarkable sparing of normal tissue. This tissue sparing effect (TSE) can also be influenced by the method of delivery. Microbeam radiotherapy creates this TSE in various organs through spatial and periodic alternation of the radiation dose.

Chemotherapy

The overall impact of chemotherapy on fertility is dependent upon the specific medication regimen and dosage administered. Many different classes of chemotherapeutic agents are available to clinicians, and these drugs are typically categorized as "high," "intermediate," and "low" risk for causing fertility impairment. Alkylating agents (cyclophosphamide, procarbazine, busulfan) are examples of high-risk agents, and vincristine is an example of a low-risk chemotherapeutic drug (Chapter 42).

Alkylating agents deserve further discussion. They disrupt DNA through base pair alkylation, leading to abnormal cross-bridge formation and mispaired nucleotides. Cyclophosphamide exhibits a dose-dependent effect, with dosages of 7.5-9 g/m^2 leading to impaired fertility, >10 g/m^2 gonadal injury, and > 20 g/m^2 usually causing permanent sterility. Green et al. have attempted to define levels of cyclophosphamide exposure below which azoospermia would not be seen. They coined the term "cyclophosphamide equivalent dosage (CED)," with the hope of providing patients with prognostic information about the effects of chemotherapy on

fertility. However, the authors found that there was no CED below which azoospermia was not seen in their cohort. Azoospermia has been found in 10% of those receiving a cyclophosphamide equivalent dose (CED) of < 4 g/m^2. The effects of alkylating agents can be long-lasting, with 25% remaining azoospermic, 28% oligozoospermic, and only 48% normozoospermic after 21-year follow-up, with each cumulative increase in CED of 1 g/m^2 being associated with worse recovery potential in reproductive status.

Platinum analogues similarly damage DNA and can interfere with replication, leading to equivalent rates of gonadotoxicty, yet their effects may be less permanent. Normozoospermic patients undergoing cisplatin therapy for testicular cancer had normozoospermia (64%), oligozoospermia (16%), and azoospermia (20%) posttreatment, but 80% recovered normal spermatogenesis by 5 years post-therapy. Carboplatin has similar effects, with one study of men undergoing multiagent BEC (bleomycin, etoposide, carboplatin) chemotherapy for testicular cancer revealing a 93% and an 83% chance of recovering of normozoospermia after 2 and 4 cycles respectively. Antimetabolites, vinca alkaloids, and topoisomerase inhibitors can have some gonadotoxicity, but their impact is more attenuated.

In addition to the individual drugs discussed above, multiagent regimens can also have a significantly adverse impact on fertility. MOPP therapy (mechlorethamine, oncovin, procarbazine, prednisone) in the setting of Hodgkin lymphoma typically leads to azoospermia in 85-90% of patients undergoing >3 courses, with additional common sequalae of low testosterone and gynecomastia. COPP therapy (cyclophosphamide, oncovin, procarbazine,) was found to have similar fertility effects in one study, with 100% of azoospermic men persisting up to 11 years post treatment. Due to their toxicity, ABVD (adriamycin, bleomycin, vinblastine, dacarbazine) has been utilized instead, resulting in 90% recovery of semen parameters at 24 months with no azoospermia compared to alkylating agents. BEP (bleomycin, etoposide, platinum, prednisone) is a common combination therapy for treatment of testicular cancer. Semen parameters typically decrease following treatment, with one study showing less than half of patients recovering to normozoospermia at 24 months. Only 2% of patients remained azoospermic following therapy, with a history of receiving >2 cycles, abnormal baseline parameters, and concurrent radiation treatment all being predictive of poor recovery.

Surgical Therapy

Along with cytotoxic therapy, surgery can also be an iatrogenic cause of infertility. Surgery involving excision of testicular tissue, as is routinely performed on patients with testicular cancer, can result in loss of germ cell mass and reproductive potential. Oncologic pelvic surgery involving excision of the prostate gland or bladder results in a disruption of the excurrent ductal system and male infertility. Retroperitoneal surgery for colon cancer and retroperitoneal lymph node dissection (RPLND) surgical procedures can result in disruption of the lumbar sympathetic structures as well as the hypogastric/pelvic plexus that contain the sympathetic outputs driving seminal emission and ejaculatory function. RPLND for testicular cancer is a significant offender due to damage incurred to the hypogastric plexus, which leads to retrograde or anejaculation in 89% of cases. However, nerve sparing protocols have improved antegrade ejaculation outcomes up to 99% in chemotherapy-naïve patients and up to 89% of those patients who received chemotherapy. Pelvic surgery for prostate, bladder, and colorectal cancers can also damage parasympathetic nerves causing either temporary or permanent erectile dysfunction. The vas deferens is also intimately intertwined with this anatomy and can be unintentionally injured during these surgical cases. Even if spermatogenesis remains unimpaired, this injury can result in an obstructive azoospermia. Thus, regardless of the need of cytotoxic anti-cancer therapy, surgery alone can inadvertently lead to erectile dysfunction, resulting in impaired ejaculation and insufficient sperm counts.

Fertility Preservation

Due to the impact that chemotherapy, radiation, and surgery have on future fertility, both the American Society of Clinical Oncology (ASCO) 2018 guidelines, and the American Urological Association/American Society for Reproductive Medicine (AUA/ASRM) 2021 combined guidelines recommend sperm cryopreservation prior to the initiation of gonadotoxic therapy. This is imperative, as even a single dose of chemotherapy can compromise spermatic DNA quality and the overall quality of the sample. Most adult male patients, as well as adolescents, are capable of producing a sufficient semen sample for fertility preservation by masturbation. Semen collection can usually be successfully completed by patients, with proper coordination of care and

assurances of patient privacy at the time of collection. Given the improved access to IVF/ICSI, only very small numbers of viable sperm are required to help ensure fertility preservation. Peripubertal boys can be evaluated for the presence of Tanner stage II development, nocturnal emission, and testicular volume of 10-12 ml as indicators for the onset of spermatogenesis and their ability to provide a specimen (Chapter28).

Retrograde ejaculation may hamper the successful attainment of an ejaculated semen sample and should be suspected in men with histories of retroperitoneal surgery, low ejaculate volumes and neuropathy. A post-ejaculation urinalysis (PEU) can confirm the diagnosis with the presence of sperm in the urine. Alpha agonists such as pseudoephedrine and tricyclic antidepressants such as imipramine have a sympathomimetic effect and can be trialed in an attempt to restore antegrade function through improvement of bladder neck contraction. Sperm can also be collected from the bladder through trans-urethral catheterization, which is usually accomplished by first instilling sperm wash media into the bladder immediately before collection.

Patients with anejaculation present additional therapeutic challenges. Penile vibratory stimulation (PVS) involves application of a non-invasive vibratory device to the frenulum to facilitate induction of a spinal ejaculatory reflex. If PVS is unsuccessful, electroejaculation (EEJ), which involves electrical stimulation to the pelvis delivered via transrectal probe, can be trialed. This approach results in the nonspecific application of direct electrical current to the prostate gland and seminal vesicles to induce ejaculation. EEJ is significantly more invasive than PVS and must be conducted under general anesthesia in patients who are neurologically intact.

Patients who remain persistently anejaculatory, aspermic, or azoospermic despite the above interventions are candidates for oncologic testicular sperm extraction (onco-TESE). This approach, first describe by Schrader et al, involves surgical extraction of testicular tissue for the purpose of fertility preservation. Onco-TESE is also considered an invasive surgical approach, with sperm extraction success rates at approximately 50% based on several studies. Unfortunately, fertility preservation options for pre-pubertal cancer patients are limited and reliant upon clinical trials to develop investigative approaches (Chapter 46).

Barriers

While barriers to fertility preservation include financial, logistical, and cultural factors, one of the most glaring factors is the role of a provider. The greatest predictor of preservation is a physician recommendation. The diagnosis of cancer coincides with significant stress and a focus on the diagnosis and initiation of oncologic treatment. This focus can lead oncologists to be less likely to refer patients for fertility preservation care, particularly in the setting of an aggressive cancer. Physician, nurse practitioner, and nurse discomfort in discussing fertility preservation with patients can also lead to low patient referral rates for sperm cryopreservation. Provider bias may also play a role, as patients with lower median income, worse prognosis, or more advanced age are all less likely to be provided counseling.

Patient factors can also play a role in fertility preservation. Assisted reproductive techniques (ART) can be expensive processes and can dissuade those without sufficient financial resources to preserve their fertility. Access to a facility where appropriate sperm cryopreservation in the midst of acute oncologic care can also present a physical barrier to care—this is particularly true at some rural and pediatric hospitals. Finally, some patients have religious or cultural beliefs that can result in objections to masturbation, sperm cryopreservation, or other facets of assisted reproductive techniques.

Fertility Preservation Programs

Despite the numerous barriers that exist, patient counseling can have a dramatic impact on rates of cryopreservation. Dedicated fertility preservation programs consist of multidisciplinary teams of oncologists, urologists, reproductive endocrinologists, geneticists, nurses, social workers, and mental health professionals providing fertility services to these patients. The implementation of formalized programs has been shown to increase consultation rates by 2.4-fold, with one study even showing a 6-fold increase in fertility preservation. A patient navigator acting as a single point of contact can also help patients access and receive fertility preservation care more efficiently while minimizing patient stress in the process.

Conclusions

The cytotoxic and surgical management of cancer can drastically impact fertility potential. While effective means for sperm cryopreservation exist, significant barriers continue to limit patient access. The establishment of dedicated, multi-disciplinary fertility preservation teams can help mitigate these barriers and facilitate successful implementation of fertility preservation care.

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Chapter 48
What is the role of the penis in male reproduction?

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Like in most mammals, the penis is an integral part of the male sexual function with one main reproductive property: deliver semen into the female reproductive tract. Herein, we discuss the basic anatomy and physiology of the male penis along with its role in reproduction and what pathologies may interfere with this process.

Penile Anatomy

Penile shaft: Corpus cavernosum and Tunica albuginea

The human penis is a unique structure composed of several fascial layers that surround a pair of corpora cavernosa and a single corpus spongiosum. The two corpora cavernosa prolong proximally as the crus and attach to the pubic arch at the level of the ischial tuberosity, while distally the septum that divides the two corpora throughout the penis becomes permeable allowing for blood communication between the two. The paired corpora cavernosa are enveloped by a thick stretchable membrane: the tunica albuginea. The tunica albuginea is composed of two layers: an outer longitudinal and an inner circular. The bi-layer structure is essential to normal erectile function as it gives the penis great flexibility, strength, and rigidity. Emissary veins that run between the two layers piercing the outer layer obliquely are compressed during tumescence. As the corpora fill with blood, venous outflow is obstructed to allow blood trapped in the penis which is necessary to generate the penile rigidity for vaginal penetration.

Glans penis and urethra: Corpus spongiosum

Sheathed by a tunical layer like the corpus cavernosum, the corpus spongiosum houses the urethra. Distally the corpus spongiosum becomes the glans penis, which is rich in nerve endings of the neuronal bundle. Proximally, at the level of the bulbar urethra, the

corpus spongiosum is enveloped with the ischiocavernosal and bulbospongiosal muscles that compress the spongiosum and penile veins to increase engorgement of the spongiosum and glans. During orgasm rhythmical contraction of these muscles function in expulsion of semen outside the urethra.

Arteries, veins and lymphatics

The main arterial blood supply to the penis is the internal pudendal artery, which is the final branch of the anterior trunk of the internal iliac artery. After crossing the urogenital diaphragm, the pudendal artery which runs through Alcock's canal gives off a branch to the perineum and becomes the common penile artery. The latter divides into three branches, namely, the dorsal, cavernosal and bulbo-urethral arteries. The cavernosal arteries are helical, tortuous arteries that supply the sinusoids and trabecular tissue in the corpus cavernosum during tumescence. The bulbourethral artery supplies the penile bulb proximally, the corpus spongiosum and the glans penis. Lastly, the dorsal artery which runs under Buck's fascia between the dorsal vein and dorsal penile nerves on the dorsum of the penis also supplies the glans penis and arborizes with the bulbourethral artery at the glans. Through cavernous branches and retrograde flow through the glans, the dorsal artery also supplies the urethra and the corpus spongiosum along the penis.

Emissary veins which pierce the tunica albuginea of the corpus cavernosum and spongiosum at an angle drain the sinuses into the deep dorsal vein during detumescence. The distal two thirds of the penis emissary veins of the corpora spongiosum drain into multiple circumflex veins which course around the corpus cavernous from the corpus spongiosum and drain into the deep dorsal vein. Multiple emissary veins join at the proximal one third of the penis to form cavernous veins that drain into the internal pudendal vein.

Hemodynamics, innervation and ejaculation

The innervation of the penis is modulated by autonomic (sympathetic and parasympathetic) and somatic (sensory and motor) inputs. Sympathetic and parasympathetic nerves from the spinal cord merge to create cavernous nerves. The sympathetic trunk simulation causes detumescent while stimulation of the pelvic plexus rich in parasympathetic causes erection. Interconnected nerve endings rich in nitric oxide (NO) cause vasodilation of sinusoids and increase blood flow to the penis. The compression of emissary veins limits venous outflow to maintain erection. Sensory information is carried through

somatic nerves especially at the glans (profuse nerve endings) during intercourse. At climax, rigid erection and ejaculation are initiated by the firing of pudendal nerve contracting the bulbocavernosus and ischiocavernosus in an alternating, rhythmic manner.

Ejaculation is divided into 2 parts: emission and expulsion. The first involves contraction of the bladder neck as the seminal vesicle and prostrate deposit semen in the bulbar urethra. This mechanism prevents retrograde ejaculation of sperm. The second part is expulsion of the semen outside the penis into the female vagina by the contraction of the bulbospongiosis muscle.

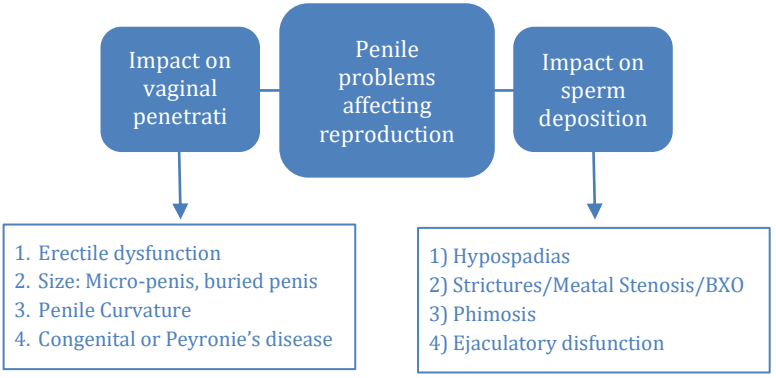

Figure1: General scheme for etiology affecting Penile reproductive function

Penile reproductive function is intact if erections are satisfactory for coitus and semen deposition occurs into the female reproducetive tract. Any deterrence to this may decrease reproductive success. (Fig.1)

Erectile Dysfunction (ED)

ED is defined as the inability to attain and/or maintain penile erection sufficient for satisfactory intercourse (Chapter 50). Whether it is due to vascular, neurogenic, psychogenic, endocrine, or medications, ED that results in erections that are not rigid enough to provide vaginal intercourse will impact natural reproduction.

Prepuce (foreskin)

Phimosis*:* inability to retract the foreskin due to adhesions between the inner or mucosal prepucial skin and the glans. When severe enough, it can be associated with pain and skin tearing during intercourse which may lead to difficulty with vaginal intercourse and subsequent fertility. Phimosis can be secondary to Lichen Sclerosis formerly known as balanitits xerotica obliterans (BXO) that is also associated with urethral strictures. If severe enough narrowing is present, urethral strictures can decrease the force of ejaculate; this may contribute to infertility.

Micro penis

A penis with a stretched length that is 2 standard deviations below the average for age. The most common cause is hypogonadotropic hypogonadism. Some etiologies could be a deficiency of testosterone secretion, or defect in testosterone action or idiopathic. Micropenis can cause difficulty with penetration and deposition of sperm within the vagina.

Buried penis

The penis is buried in pre-pubic fat. This is often associated with obesity. Another similar condition is trapped penis from scarring after penile surgery.

Abnormal penile orientation

Penile curvature occurs along the vertical (dorsal vs ventral) or horizontal axis (lateral) and is either congenital or acquired. 25 % of men have an angulated erect penis. While in most patients some penile curvature is not problematic, in others it might be severe to impact body image, sexual satisfaction and affect fertility. Curvatures of the penis can be acquired or congenital.

Congenital penile curvature is due to disproportionate lengths of corporal tissue. While congenital curvature is often ventral, it can be in any direction (dorsal, lateral, or multi-planar) Ventral curvature can be associated with hypospadias and in this case it is often referred to as chordee. When severe enough, congenital curvature can make intercourse difficult, painful, or impossible.

Peyronies'disease (PD) is an acquired curvature of the penis. It is an inflammatory condition of the tunica albugenea. This scar is caused by imbalance between collagen deposition and degradation at the tunica that makes it inelastic. PD is thought to be caused by repeated micro-traumas to the tunica. The curvature occurs

ipsilateral to the plaques, or occasionally as an hourglass deformity. It can cause penile pain, shortening and deformation. ED is associated with PD, which might be due to veno-occlusive dysfunction. As with congenital curvature, severe penile angulation itself can prevent sexual intercourse.

Hypospadias

It is one of the most common congenital anomalies in men that usually encompasses three conditions with variable degree of each: abnormal urethral meatus ventrally, penile curvature ventrally, and partially covering foreskin dorsally. Fortunately, the less severe, distal form is more common. Moreover, proximal hypospadias shows an associated worse curvature in patients than distal hypospadias.

Abnormal anatomic location of the urethra in hypospadias may result in deposition of semen too distal in the female reproductive tract. Although, men would have relatively acceptable seminal parameters, this abnormality may impact the reproductive function of the penis.

Additionally, hypospadias is associated with other abnormalities such as cryptorchidism and disorder of sexual differentiation (DSD). In fact, in a patient with unilateral or bilateral cryptorchidism, especially if associated with proximal hypospadias, the AUA guidelines recommend a routine DSD evaluation. This includes detailed medical history, physical examination, and laboratory and imaging examinations that are required collectively for diagnosis and sex identification. Thus, the reproductive function of the penis may be deterred not only by hypospadias as a malformation but also by its associated disorders.

Urethral stricture

It is an abnormal narrowing of the urethra that can develop throughout the length of the male urethra. The etiologies include idiopathic, iatrogenic, external trauma, infection and inflammation such as lichen sclerosis. Patients present with voiding lower urinary tract symptoms (LUTS) and painful ejaculation. A stricture may cause poor ejaculation.

Ejaculatory dysfunction

Retrograde ejaculation: it is an uncommon cause of infertility, can be congenital, acquired or idiopathic. Most common causes include use of medication such as alpha blocker and post-surgical transurethral resection of the prostate (TURP). Examination and processing of post

ejaculate urine can lead to successful recovery of viable sperm that can be used to achieve pregnancy with assisted reproductive technologies.

Anejaculation: can be neurogenic (acquired or iatrogenic) or idiopathic. Spinal cord injury is the most common cause of neurogenic anejaculation. Retroperitoneal lymph node dissection is a surgical procedure involving dissection along the sympathetic chains and hypogastric plexus. Any injury to these nerves post-operatively can affect efferent stimulation for seminal vesicle emission and bladder neck closure causing anejaculation.

Ejaculatory duct obstruction: sperm and seminal vesicle fluids enter the prostatic urethra through the ejaculatory duct located on the sides of the verumontanum at the level of the utricle.

Obstruction can be congenital or acquired, it can be due to a persistent utricle cyst, congenital atresia of the ducts, seminal vesicle calculi, iatrogenic post-surgery or post inflammatory. Patients may present with hematospermia, painful ejaculation or infertility with low volume ejaculate on spermogram.

A functioning penis plays a crucial role in natural procreation to allow effective delivery of sperm to the female reproductive tract. Thanks to the advances in assisted reproductive technologies (ART), the dysfunctional penis is no longer considered a limiting factor for procreation as long as the male has well-functioning testes (see chapter 35).

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Chapter 49
The potential reproductive effects of male circumcision

Nahid Punjani and Philip S. Li

Foreskin and male circumcision

The foreskin, also known as the prepuce, is the double layer of skin covering the male phallus protecting the glans penis and urethral meatus. The foreskin length is variable and flexible, therefore, its coverage in both the flaccid and erect state varies. While it is attached to the glans at birth, it is generally retractable by adolescence and adulthood. The outer preputial skin is contiguous with the penile shaft, but the inner layer is more similar to a mucous membrane. As a result, the inner surface of the foreskin contains a higher density of Langerhans' cells, which may be implicated in predisposition to certain genitourinary conditions.

Male circumcision (MC), or removal of the foreskin, is among the oldest and most widely performed male procedures surgically, culturally and religiously. An estimated one-third of males are circumcised worldwide with evidence dating back to 2300 BC with Egyptian wall paintings depicting men without foreskin. MC was performed for religious or cultural practice in many cases, but its use and indications have expanded over time. Currently, voluntary medical male circumcision (VMMC) is performed for various reasons, such as individual preference and cosmesis, and critical medical indications such as recurrent infections, phimosis, discomfort, and HIV prevention.

To date, a significant body of research, including large randomized controlled trials, have focused on MC's role in HIV prevention. In 2007, The World Health Organization (WHO) and the Joint United National Program on HIV/AIDS (UNAIDS) endorsed VMMC in countries with low rates of MC and high rates of HIV as a critical strategy for HIV prevention (up to 60%). Such programs continue to expand, including broadening indications for VMMC to adults and adolescents, and its role in infants is currently being investigated. Furthermore, VMMC is also supported by the U.S.

Centers for Disease Control and Prevention, given its numerous benefits for HIV prevention and other health outcomes.

Effects on reproductive health

The primary evidence of the impact of MC and its reproductive effects arises from its potential ability to reduce genitourinary infections. For some infections, the evidence is compelling about the reproductive impacts, and in other cases, the data remains deficient. A summary is presented in Table 1.

Human Immunodeficiency Virus (HIV)

HIV is a member of the *Lentivirus* family of retroviruses and consists of two sub-types, HIV-1 and HIV-2. The virus has claimed over 30 million lives worldwide and is transmitted through various bodily fluids, with sexual transmission remaining the most common transmission source. Infection is believed to occur secondary to smaller lesions or ulcerations of the genital and rectal mucosa with a virus targeting host antigen-presenting cells (APCs) via binding of CD4 and chemokine receptors, resulting in depletion of the immune system. According to data from three clinical trials in Africa, MC can reduce a man's risk of acquiring HIV infection by over 50% to 60% during sex with HIV-infected female partners. Mechanistically, this impact is hypothesized to occur through surface area reduction, including limiting Langerhans' cells, alteration of microflora, increased viral susceptibility and permeability of the inner foreskin (Fig. 1), and variable tissue structure permitting viral entry. The role of HIV in male fertility includes a reduction in semen quality such as motility, morphology, and increased risks of aneuploidy. HIV in the testis is also linked to chronic orchitis, which may potentially lead to testicular failure, impaired semen quality and hypogonadism. Finally, treatment for HIV in anti-retroviral therapy has also been suggested to impair semen quality.

Human Papilloma Virus (HPV)

HPV is a DNA virus from the *Papillomaviridae* family with over 150 subtypes, of which multiple are cancer-causing, commonly HPV 6, 11, 16, and 18 in humans. Typically, these present as anogenital warts, with the majority being asymptomatic and resolving without intervention. While the evidence is variable, MC has a suggested protective effect in HPV infection and transmission in many reports. Potential etiologic mechanisms include limiting viral access as the inner prepuce is non-keratinized, and therefore, more susceptible to

injury and viral infection during intercourse, reduction in surface area for viral entry, exposure to areas that may be otherwise missed for HPV detection, and finally, reduction of the moist environment which may assist in HPV infection and transmission. HPV infections have been described to impact fertility, including impacts on semen parameters such as sperm concentration, motility and morphology, production of anti-sperm antibodies, and the virus may be transmitted at the time of fertilization, potentially resulting in failed implantation. MC has also been shown to reduce the risk of HPV-related cancers such as penile cancer in men and cervical cancer in female sexual partners.

Herpes Simplex Virus (HSV)

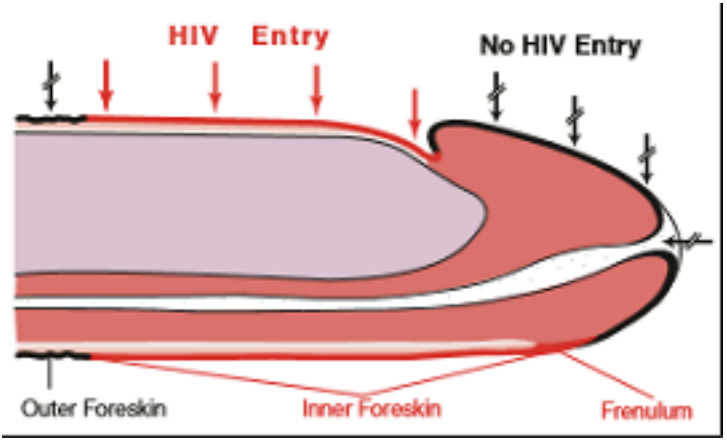

Figure 1. Illustration of how the inner foreskin permits HIV viral entry (https://circumcisionandsex.wordpress.com/2017/01/04/biological-and-evolutionary-plausibility-of-the-benefits-of-circumcision)

HSV arises from the *Herpesviridae* family and includes both HSV-1 and HSV-2. The former has generally been associated with non-genital lesions, and the latter is the more common culprit for genital herpetic lesions. In most cases, infection is limited to beefy red ulcers with possible lymphadenopathy but may result in more severe systemic symptoms and central nervous system involvement. The data for transmission reduction of MC in HSV is variably reported in the literature. The mechanistic etiology includes the removal of epithelial, dendritic, and Langerhans' cells, which permit viral entry.

The role of HSV in male fertility is similarly controversial. Animal studies have suggested impacts on sperm morphology and germ cell apoptosis. In humans, the infection has resulted in some instances of impaired semen quality, such as reduced sperm count and morphologic changes. These changes are thought to occur secondary to a viral gametotoxic effect on spermatogenesis, viral sperm DNA damage, and cross-reactivity, thereby disrupting spermatogenesis and/or an inflammatory response resulting in prostatic dysfunction and changes to seminal fluid.

Syphilis, Chancroid, Gonorrhea and Chlamydia

Syphilis is caused by *Treponema pallidum*, a spirochete bacteria, and has approximately 12 million cases each year globally. It may produce primary localized painless genital lesions, secondary systemic symptoms and may result in latent syphilis with cardiovascular and neurologic impacts. The evidence supporting the role of MC in infection reduction is controversial but is mechanistically similar to other conditions and are thought to occur due to reduction of surface area and susceptibility of micro-tears which may act as sites of bacterial infection. Limited data have been reported on the impacts of syphilis on semen quality. However, syphilis may result in epididymal obstruction or impaired testicular function from tertiary syphilis and is well known to have a role in spontaneous abortion and stillbirth.

Chancroid arises from *Haemophilus ducreyi*, a gram-negative bacterium, and usually results in painful genital sores as well as inguinal lymphadenitis. The evidence for MC in chancroid reduction is limited and controversial, and the evidence about its impacts on fertility is scant.

Gonorrhea is caused by the gram-negative diplococcus, *Neisseria gonorrohae,* and Chlamydia the gram-negative bacteria, *Chlamydia trachomatis*. Generally, these infections are uncomplicated but may result in severe sequalae with more systemic symptoms and genitourinary tract complications of urethritis and epididymoorchitis. Again limited and conflicting data exists for infection prevention by MC, with more data favoring a reduction in gonorrohea infection.

MC does reduce the risk that a female partner will acquire a new syphilis infection by up to 60%. Furthermore, it is hypothesized that the impacts on fertility may result from the genitourinary complications of urethritis and epididymoorchitis.

Table 1. Summary of the impact of male circumcision on male genitourinary infections and fertility

Infection	MC and infection		MC and infertility	
	Role in infection prevention	Mechanism	Role in infertility	Mechanism
HPV	Favorable association	Limits injury of the non-keratinized inner prepuce epithelium Reduces surface area for infection Eliminates environment for HIV transmission	May help decrease the effect of HPV	HPV virions may harm semen parameters Reduce possible anti-sperm antibody production Reduce transmission during oocyte fertilization
HIV	Decreased risk of heterosexual HIV transmission in areas with high viral incidence	Reduces injury to non-keratinized inner prepuce epithelium Reduction of Langerhans cells which act as a site of viral entry	May help mitigate the negative effects of HIV	Direct viral impact on sperm quality Possible testicular failure from chronic orchitis Anti-retroviral therapy impacts to semen parameters
HSV	Some impact reported	Removal of epithelial, dendritic, and Langerhans cells, which facilitate viral replication	Controversial evidence of the link between HSV and fertility. Possible benefits derived from MC	Direct viral toxic effects impairing semen parameters and causing sperm DNA damage Inflammatory response altering seminal fluid Cross-reactivity to self disrupting spermatogenesis
Syphillis	Suggestions of protective effects	Removal of surface for pathogen replication Reduces micro-trauma and subsequent bacterial infection	May be protective through reduction of long-term genitourinary tract complications	Severe disease may lead to epididymal obstruction or testicular lesions impacting testicular function
Chancroid	Possibly protective	Reduces surface area for infection	Limited data to draw conclusions	N/A
Gonorrohea & chlamydia	Limited data for protective role	Impairs moist environment for bacterial replication Reduces micro-trauma and subsequent bacterial infection	May be protective through reduction of long-term genitourinary tract complications	Urethral strictures Epididymo-orchitis which may result in impaired testicular function and spermatogenesis

MC: male circumcision, HPV: human papilloma virus, HIV: human immunodeficiency virus, HSV: herples simplex virus, N/A: not availablena:

Conclusion

In conclusion, the role of MC and reproduction continues to be elucidated. Its protective role is anchored in genitourinary infection reduction. In addition, there is reasonable and compelling evidence to support that MC reduces some infections, such as HIV and HPV. However, with limited variable and controversial data, further prospective studies are needed to explore these relationships and the overall reproductive impact.

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Chapter 50
How do erections occur? How common is erectile dysfunction, what is its etiology, and how do you evaluate men with this problem?

Logan B. Galansky, Phillip M. Pierorazio and Arthur L. Burnett

How do erections occur?

Penile erection requires an elaborate orchestration of neural, vascular, and hormonal processes in the proper psychological setting. Beginning with tactile stimulation of the penis or mental arousal, penile erection is a spinal reflex that is triggered by peripheral or central nervous stimuli. Erection is mainly mediated by parasympathetic nerve fibers located in the pelvic ganglion, which course via the cavernous nerve to the spongy vascular chambers of the penis, known as the corpora cavernosa.

Once a neural signal is transmitted, the process of tumescence begins with the filling of the corpora cavernosa with blood to create a rigid organ usable for sexual intercourse. Erections are initiated and maintained by two vascular processes: relaxation of the arteries of the corpora cavernosa to allow improved inflow of blood and increased resistance of outflow venous channels to maintain tumescence. The increased inflow is a complicated neurohormonal process, whereby messenger molecules, primarily nitric oxide, but also cGMP, cAMP, and others, are released by local neurons leading to smooth muscle relaxation, arterial dilation, and augmented blood flow. Increased resistance to outflow is a passive process wherein the venous channels are compressed against the tunica albuginea, the fibrous covering of the corpora cavernosa, by the expanding penile tissue.

Pelvic floor musculature is also key in the erectile response. The bulbospongiosus and ischiocavernosus muscles surround the corporal bodies at the base of the penis. These muscles contract in response to the bulbocavernous reflex during sexual arousal and help produce the rigid erection phase.

How common is erectile dysfunction?

Erectile dysfunction (ED) is defined as, "an impairment in the arousal phase of [the male] sexual response" with "consistent or recurrent inability to attain and/or maintain penile erection sufficient for sexual satisfaction, including satisfactory sexual performance." (AUA Guidelines, 2018).

The prevalence of ED has been reported by numerous sources, with approximately 20 percent of adult men classified as having ED. It is estimated that up to 150 million men globally have ED. Prevalence also increases with age. For men less than 40 years old, the worldwide rate of ED ranges from one to nine percent, but does appear to be increasing over time. 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. The development of ED has been associated with other medical conditions and risk factors, namely hypertension, diabetes mellitus, dyslipidemia, smoking, and cardiovascular disease.

What is its etiology?

Erectile dysfunction can manifest in a variety of ways and have many physiologic causes. Patients may be unable to achieve a complete erection, or to maintain an 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 and sustain an erection.

The causes of ED comprise five specific categories: (1) vasculogenic, due to either arterial or venous problems, (2) neurogenic, (3) endocrinologic, (4) medication-induced 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. Metabolic syndrome, a constellation of risk factors for heart disease, diabetes, and stroke, including hypertension, increased fasting glucose, central obesity, and dyslipidemia, has been identified as an increasingly common cause of ED.

Neurogenic causes are estimated to make up 10 to 19% of ED and 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. While most men with ED do not have hypogonadism, studies have shown that once testosterone levels drop below a threshold of roughly 230 ng/dL, men can begin to experience ED. This may be due to the local effects of testosterone on penile tissue during erection.

Many cases of ED are medication-related. The most common culprits are anti-hypertensive medications, especially beta-blockers, that lower systemic blood pressure and lead to decreased blood perfusion to the penis for erection to occur. Other medications, such as many classes of psychiatric medications, can decrease libido or reduce levels of male sexual hormones (i.e. testosterone) and consequently lead to ED. Moreover, it is important to educate any man undergoing androgen deprivation therapy (ADT) that ED is a well-established potential side effect.

Psychogenic causes are believed to play a role to some extent in all cases of ED (often combined with another etiology). The most common psychogenic causes are depression, anxiety, and stress, although various psychiatric disorders can contribute to ED. Ultimately, mental health problems, emotional stressors, and inter-personal conflicts can have a negative impact on erectile function either primarily or secondarily.

Finally, 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, which can hinder the ability to achieve erection.

How do you evaluate men with this problem?

Shared decision making and an individualized approach to care are fundamental tenets for evaluating and treating ED. As such, all men presenting with symptoms of ED should undergo a comprehensive evaluation of medical, surgical, and psychosocial history, physical examination, medication reconciliation, and laboratory studies with additional specialized testing as indicated.

In 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 Fig. 1.

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.

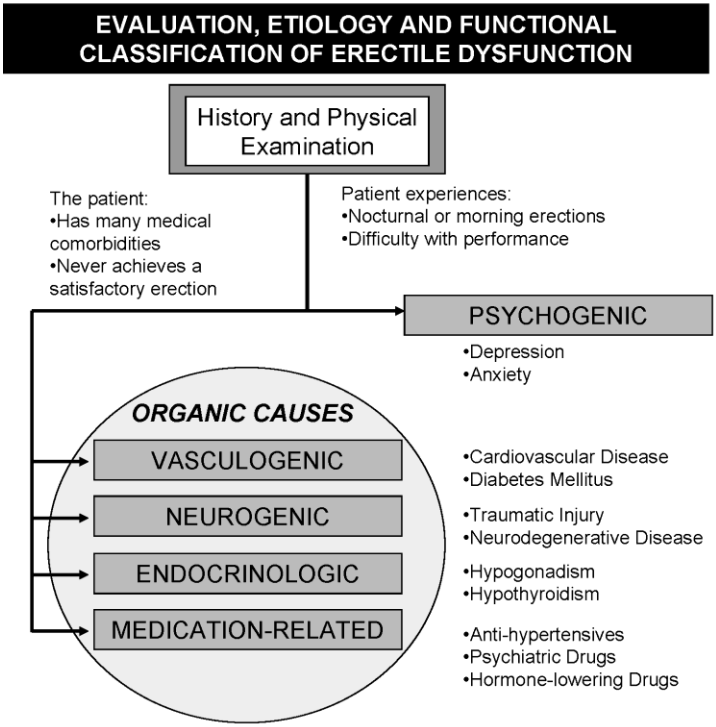

Figure 1. Evaluation, Etiology and Functional Classification of Erectile Dysfunction (ED). Common examples of disease states in each category are listed adjacent to each.

Questionnaires such as the Sexual Health Inventory for Men (SHIM), International Index of Erectile Function (IIEF), and Erection Hardness Score (EHS) 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. Additionally, discussing any psychological or interpersonal issues that may be contributing to the patient's ED symptoms is crucial to obtaining a thorough sexual history.

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 investigating 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.

Furthermore, 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. ED has come to be understood as an early indicator of possible cardiovascular or neurovascular disease, with some studies finding that diagnosis of moderate ED can precede presentation of serious systemic vascular disease by 2-3 years. Thus, determining the presence of these potentially modifiable factors can help patients not only improve their erectile function, but also decrease their risk of having a significant cardiovascular event (heart attack or stroke) in the future.

Physical examination should involve a careful 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 systematic vascular and neurological examination including palpation of peripheral pulses, abdominal exam for abdominal aortic aneurysm, inspection of the lower extremities for loss of hair growth, and neurologic exam for reflexes and neuropathy can help identify vasculogenic or neurogenic causes of ED.

In certain cases, additional laboratory and physiological tests may be helpful to define the cause or measure the severity of ED. For instance, serum lipid levels and hormone levels can help identify certain cardiovascular and endocrinologic etiologies of ED. In men

diagnosed with ED, it is recommended that morning serum testosterone levels be measured.

Further specialized testing is recommended only if it will influence ED management. Penile duplex ultrasound is the current gold-standard to determine whether or not there is adequate blood flow to the penis. Nocturnal penile tumescence and rigidity testing, office intracavernosal injection, and cavernosometry are other adjunctive tests that can aid in the evaluation of complex ED. Overall, many evaluations of ED are complete without the need for complicated testing – a detailed history and physical examination are often sufficient before initiating ED treatment.

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Chapter 51
How to treat erectile dysfunction?

Wael S. Almajed, Gilad A. Hampel and Wayne J.G. Hellstrom

Introduction

Management of erectile dysfunction (ED) varies considerably depending on the etiology. Following a focused evaluation of the ED patients (Chapter 50), 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 (e.g. those with Peyronie's disease, pelvic injury, endocrine disorders, complicated psychiatric disorders. and young males unresponsive to oral agents).

Nonpharmacologic management

Erectile dysfunction could be a sign of cardiac and peripheral vascular disease. Thus, lifestyle modifications that improve cardiovascular health may also improve erectile function. It has been demonstrated that men who lose weight and enhance their physical activity had significant improvement in erectile function. In contrast, men with higher body mass index (BMI) and a sedentary lifestyle are at increased risk to develop ED. In addition, tobacco use increases the risk of ED in a dose-dependent fashion. Other risk factors for ED include hypertension, dyslipidemia, and type 2 diabetes mellitus.

Medications can also have a profound effect on sexual function. Studies have shown that up to 25% of ED presentations are associated with medications. These adverse effects from common medications can range from decreased blood pressure, hormonal alterations, diminished sexual arousal, or central suppressive effects (Chapter 50). Treatment of hypertension is one of the most common causes of medication-induced ED, as nonspecific □-blockers and thiazide diuretics are known causative agents. Other common medications associated with ED include antiandrogens, antidepressants, and other psychotropics. When a medication is identified as the potential cause for ED, cessation should be

considered. If a medication is essential, substitution with a different medication may be considered. For example, use of calcium channel blockers and angiotensin- converting enzyme inhibitors are alternative antihypertensive agents, as both have decreased negative effects on sexual function. Moreover, other antidepressant medications, such as bupropion or venlafaxine, may also have decreased inhibitory effects on sexual function. Care should be taken to first communicate such changes with the patient's primary care provider to avoid the risk of discontinuing essential medications.

Psychosexual counseling

Psychological counseling has been used with success in various subsets of men with ED. In general, men with psychogenic ED are the main beneficiaries of sexual counseling. These men are generally found to be physiologically normal in terms of erectile function but may suffer some cognitive issues that affect sexual function. Interventions include systematic anxiety reduction, interpersonal therapy, sex education, and couples' therapy. Counseling is often utilized in conjunction with pharmacologic strategies to improve outcomes, especially in unmotivated men.

Vacuum erection device

One nonpharmacologic management strategy that aims to physically produce an erection, as opposed to modifying risk factors, is the vacuum erection device (VED). The mechanism of VED creates a negative pressure on the penis to enhance blood engorgement into the cavernous spaces, thus inducing an erection-like effect. VED is often used in conjunction with a penile constrictive band or ring to maintain an erectile state during intercourse. Advantages of the device include its low cost and ability to produce a rigid erection sufficient for intercourse, including engorgement of the glans. Common drawbacks include pain and bruising at the band site, decreased ejaculate volume, including anejaculation in some cases, caused by the constrictive band, and lower penile temperature and sensation. Those negative side effects have led to a low satisfaction rate (30%-70%) and a high discontinuation rate (up to 60%) after one year.

Pharmacologic management for ED

Phosphodiesterase type-5 inhibitors

PDE-5 inhibitors remain the mainstay treatment for most men with ED due to their attributes of effectiveness, simplicity, and non-invasiveness. In 1998, sildenafil citrate (Viagra, Pfizer, New York, NY, USA) was approved by the FDA. Since then, vardenafil (Levitra, Bayer Schering Pharma AG, Berlin, Germany) and tadalafil (Cialis, Eli Lilly, Indianapolis, USA) were approved for use in 2003, followed by avanafil (Petros Pharmaceuticals, New York, NY, USA) in 2012. All four 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, causing detumescence. PDE-5 inhibitors inhibit this enzyme and maintain cGMP levels, thereby promoting an erection (Fig. 1).

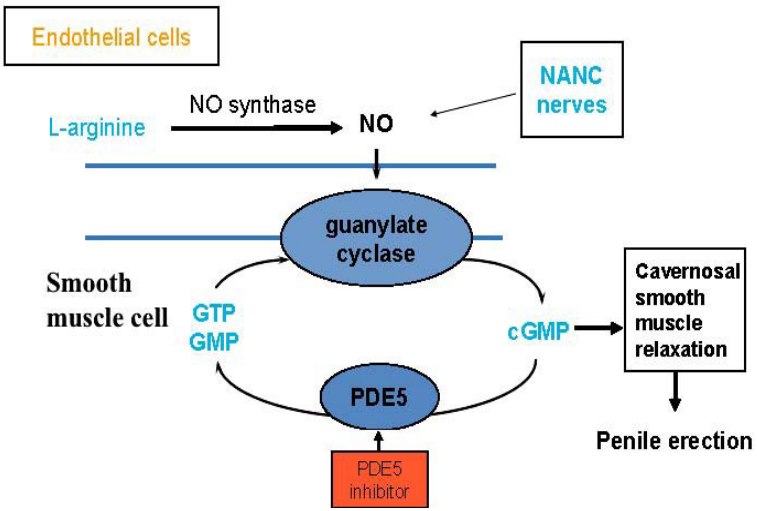

Figure 1. The role of nitric oxide (NO) and cGMP in the physiology of erection and PDE-5 inhibitors in promoting tumescence.

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 nonarteritic 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 affect the QT interval on electrocardiogram. Therefore, some men taking antiarrhythmics should avoid vardenafil. PDE-5 inhibitors are also contraindicated in men taking nitrates because of the risk of a pronounced drop in blood pressure. Men taking α-adrenergic antagonists for benign prostatic hyperplasia 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 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 all causes of ED.

Intracavernosal or transurethral vasoactive 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 exploited since 1982. These vasoactive agents, which include alprostadil, papaverine and phentolamine, are injected directly into the cavernosal tissue to produce an erection. Alprostadil (Prostin VR) works by increasing intracellular cyclic AMP levels and decreasing intracellular calcium. It is the only FDA-approved injectable medication for ED and is available under the trade names Caverject (Pfizer, New York, NY, USA) and Viradel/Edex (Schwarz Pharma, Milwaukee, WI, USA). After injection, the medication is locally metabolized by 96% within 60 minutes and produces a full erection at doses of 10 to 20 μg in 70% to 80% of men with ED.[1] Papaverine is a nonselective PDE inhibitor that increases intracellular cAMP and cGMP levels. Phentolamine is an α-adrenergic antagonist that increases presynaptic norepinephrine levels. These medications are used alone or in combination for injection into the cavernosal tissue. 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.

Intraurethral suppositories, which were developed with the hope of avoiding the invasive nature of intracavernosal needle injection, are another way of administering vasoactive agents into the erectile bodies. Specifically, the vasoactive agents are absorbed into the corpus cavernosum through the mucosal lining of the surrounding corpus spongiosum. A synthetic formulation of alprostadil was developed and approved by the FDA in 1996 as MUSE (Medicated Urethral System for Erection; MEDA Pharmaceuticals, Somerset, NJ, USA). The responder's rate for MUSE is approximately 50%, and men must properly dispense and manually distribute the medication into the penis to optimize success.

Testosterone **replacement**

Studies have demonstrated 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 (TRT). These men on TRT should be cautioned regarding the risks of hormonal replacement, including erythrocytosis and possible effects on the prostate. These men should be monitored while on therapy with semi or yearly digital rectal examination (DRE), measurement of serum prostate specific antigen (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 TRT. Recent studies have reported that TRT is likely safe in men with a history of confined prostate cancer; however, patients should be well-informed and judicious about follow-up. Testosterone replacement therapy can be delivered through injections, transdermal gels and patches, oral formulations, nasal 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 unamenable 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 the two-piece device, the scrotal pump and reservoir are 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 such as infection or malfunction of the device may necessitate revision or 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 with documented arteriogenic insufficiency diagnosed with pelvic angiography. Successful revascularization in these patients can result in normal erectile function in the majority of men.

Experimental Therapies

In recent years, there has been an increasing interest in finding a durable therapy for ED. Proposed treatments include platelet-rich plasma (PRP), intracavernosal stem cell therapy, and low-intensity extracorporeal shockwave therapy (Li-ESWT). According to the FDA, these therapies are experimental and need further evidence before regulatory approval.

Intracavernosal Botox injections have been recently explored and yielded subjective improvement by validated questionnaires in recent studies. Other emerging pharmacologic interventions include selective activators of maxi-K channels, guanylate cyclase activators, and nitric oxide (NO) donors such as L-arginine. Many of these preclinical animal studies have reported promising results, but they remain experimental in clinical practice at this time.

Summary

Currently, most men with ED can be safely prescribed a trial of oral PDE-5 inhibitors following a basic 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 vasoactive therapy, or advance to surgical options.

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Chapter 52
Is there an andropause, more appropriately named Late Onset Hypogonadism, and if so, what tissues are affected and how?

Mitch Kim, Roei Golan, Manish Narasimman and Ranjith Ramasamy

Even thousands of years before isolating the compound, which we now call testosterone, scholars across the globe understood that some component of the testes was important for the health and vitality of men. There is documented evidence that traces back to Roman times, of utilizing animal testicular tissues to improve the declining virility of aging men, demonstrating the collective understanding of the intimate link between gonadal failure and common symptoms of old age.

Today, it is understood that testosterone is a hormone produced in the Leydig cells of the testes and this hormone is responsible for male sexual differentiation and development; these cells are regulated by the hypothalamic-pituitary-gonadal axis (Chapter 2). Testosterone affects the entire body regulating many functions including fertility, sex drive, erectile function, bone density, fat distribution, and lean muscle mass. While it is no surprise that men with low testosterone will show impairment in these functions, a decrease in testosterone can also present symptoms of reduced energy, diminished physical performance, visual field changes, anosmia, depression, decreased concentration, and impaired memory.

Aging is associated with gender-specific hormonal changes that progressively lead to gonadal insufficiency. While all women can expect to experience menopause as they age with dramatic decline in circulating estradiol and progesterone, men do not experience a similar rapid decline in steroid production. Rather, aging men experience a decline in circulating testosterone over several decades; this is termed late onset hypogonadism (LOH), previously inappropriately referred to as andropause. LOH is associated with advanced age and characterized by symptoms of deficiency in serum testosterone. Serum testosterone levels decline gradually with age

at an approximate rate of 1% per year after the third decade of life; however, only 20% of men aged 65 years or more have testosterone levels below the normal range for young men.

LOH was first coined in 2002 and defined as a disease entity. Several societies including the American Society of Andrology, the American Urological Association (AUA), and the European Academy of Andrology have endorsed recommendations for further investigation into this clinical and biochemical syndrome. The efforts of these societies led to guidelines that accurately diagnose LOH, which were then updated and adopted by other societies, including the Canadian Society of Urology, the European Menopause and Andropause Society, and the Endocrine Society.

It is worth noting that not all academic entities have adopted the term LOH to describe this condition. The AUA and the Testosterone Panel were also committed to creating a guideline that ensures that men in need of testosterone therapy (TT) are treated effectively and safely. The Panel chose to cease the use of the term hypogonadism as it has recently been used interchangeably with the idea of low testosterone production alone. To capture the full clinical picture, the AUA has adopted the term testosterone deficiency (TD) in place of LOH, as it better describes the signs and symptoms associated with low total testosterone in addition to the state of low testosterone production itself. Thus, a patient diagnosed with TD is a candidate for TT only when he meets both criteria.

While there is consensus in the guidelines of various societies that TD is based on the presence of both abnormal laboratory measurements and clinical signs and symptoms, there is variation in the exact laboratory cut off value of testosterone concentration that they use. This discrepancy is likely due to various symptoms of TD correlating to different testosterone threshold levels. In other words, professional societies view different symptoms as the priority point to start TT. Hence, a reliable cut-off value for testosterone level is critical to accurately diagnose TD in aging men given the correlations that have been observed between TD and several more life-threatening issues.

Approximately 1-4% of total testosterone circulates in the body as free form and the rest is bound to sex hormone binding globulin (SHBG) or albumin. Since testosterone binds to albumin with low affinity, albumin-bound testosterone provides most of the bioavailable testosterone that is physiologically active. SHBG binds testosterone with high affinity, and its concentration increases with age, thereby further decreasing bioavailable testosterone. In addition, aging is also

associated with a decline in the ability of Leydig cells to synthesize testosterone, and an increase in the sensitivity of the hypothalamic-pituitary axis to be inhibited by circulating testosterone.

The 2018 AUA testosterone guidelines defined the clinical diagnosis of TD as having total testosterone concentrations below 300ng/dL recorded on two separate mornings, using the same assay from the same laboratory. This must be combined with patient interviews and physical symptoms to assess symptoms and/or signs associated with low testosterone levels, and counseling to evaluate potential risk factors. If the testosterone levels are consistently low, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) should be measured to determine if the hypogonadism is primary or secondary (Chapter 32). Secondary hypogonadism with other possible hormonal deficiencies warrants further testing for prolactin levels and magnetic resonance imaging (MRI) of the *Sella Turcica* region. The Endocrine Society has adopted these guidelines and recommends an evidence-based, individualized approach to TT in older men with TD (Fig. 1).

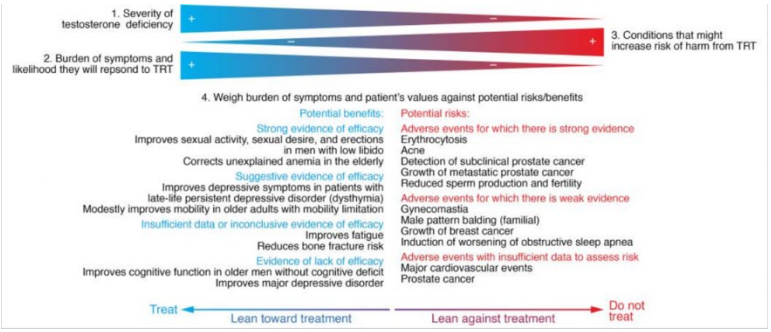

Figure 1. An evidence-based, individualized approach to testosterone therapy (TT) in older men with testosterone deficiency (TD). Bhasin S. Testosterone replacement in aging men: an evidence-based patient-centric perspective. J Clin Invest. 2021 Feb 15;131(4):e146607.

Testosterone is a steroid hormone that exerts its biological action through binding to androgen receptors located in target cells of various tissues (Chapter 3). Target tissues include sexual organs, as well as the brain, liver, muscle, skin, and bone. Commonly reported presentations of TD in aging men include diminished sexual desire, reduced semen volume, delayed ejaculation, and

erectile dysfunction with reduced morning and nocturnal erections. Affected men express decreased sense of vitality, with mood changes, decreased intellectual activity, decreased spatial orientation, depression, and anger.

Age-related TD leads to increased loss of skeletal muscle volume in conjunction with an increase in fat mass. Loss of muscle mass combined with decreases in bone mineral density, predisposes aging men to osteoporosis and subsequent fractures. TD has also been associated with metabolic syndrome in men due to increased insulin resistance and accumulation of visceral adipose tissue. Through this effect, TD may additionally contribute to the development of non-alcoholic fatty liver disease in men. Since testosterone has a stimulatory effect on erythropoiesis, TD is associated with anemias of known cause (iron deficiency) and unknown cause. Gynecomastia may also be clinically observed as both TD and increased fat lead to more unopposed estrogen that can result in a stimulation of breast tissue growth. Lastly, TD may play a role in skin alterations in aging men such as decreased epidermal skin moisture and hair concentration, skin thickness, and elasticity. However, it should be noted that these symptoms can also be manifestations of other co-morbid conditions prevalent with aging; thus, it is imperative that clinicians can exclude other diseases first before making the diagnosis of TD and begin TT.

In order to improve age-related risks for adverse health outcomes, the question of restoring the diminishing testosterone level has garnered much interest and has inevitably set the stage to develop various formulations of TT to treat TD. TT has been approved in the United States since the 1950s and over the years, various options have been developed, including formulations in topical patches and gels, nasal gel and buccal tablets, oral pills and capsules, and injections and implants. Table 1 shows TT approved by the Food and Drug Administration (USA) as of 2021.

All these options, when used according to recommendations, can restore the testosterone concentration to normal physiological range of 450-600ng/dL and relieve symptoms in most hypogonadal men. However, it is important to note that while the FDA can approve a drug for its intended use when submitted with substantial evidence of clinical benefits, the FDA does not regulate any off-label use of a drug. Clinicians are expected to make the best judgment with adherence to the guidelines to optimize the benefit-to-risk ratio of TT for each patient.

Delivery System/Drug	Brand Name	Recommended Dose Regimen	Available Format
Topical/Transdermal			
Testosterone patch	Androderm	2 or 4 mg patch/day	4 mg starting dose Do not apply the patch to the same area within 7 days Apply to back, abdomen, upper arms
Testosterone gel	AndroGel	1% gel – 50 to 100 mg of testosterone per day 1.62% gel – 40.5 to 81 mg of testosterone per day	25 or 50 mg testosterone packets Apply to shoulders and upper arms 20.25 mg testosterone, one pump actuation or a 20.25 mg packet 40.5 mg testosterone, two pump actuation or a 40.5 packet Apply to shoulders and upper arms
Testosterone gel	Testim	1% gel – 50 mg of testosterone/tube	50 mg/day starting dose Apply to shoulder and upper arms
Testosterone gel	Fortesta	2% gel 10 mg/0.5 g per pump actuation	40 mg (4 pump actuations)/day starting dose Apply to inner thighs
Testosterone gel	Vogelxo Testosterone Gel	1% gel 50 or 100 mg per tube or packet, 12.5 mg per actuation for pump 1.62% gel similar to AndroGel (1.62%)	Generic testosterone gel Generic, same as AndroGel 1.62%
Testosterone lotion	Axiron	2% lotion 30 mg/pump actuation	Start with 60 mg Apply to axilla Discontinued
Buccal/Nasal			
Buccal tablets	Striant	30 mg twice/day	Apply to gum Dislodging of tablets Discontinued
Nasal gel	Natesto	11 mg gel intranasal three times per day	Start with one actuation (5.5 mg) into each nostril total 11 mg Apply to nose three times per day

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Chapter 53
Are there reproductive consequences associated with advanced paternal age?

Peter Chan and Bernard Robaire

Introduction

There is a progressive increase of paternal age at conception across the world. The birth rate among 35 to 49 year old American men in 2015 was 69.1 per thousand compared with 42.8 per thousand in 1980. Other countries have reported a similar trend that appears to be consistent across all races, ethnicities, regions and level of education. The impact of paternal aging on reproduction and offspring health has long been an important subject in Andrology.

Model to study aging-associated changes in male germ cell

A wide range of animal models, ranging from insects to worms, birds, fish and mammals, has been used to investigate the effects of paternal aging on male reproduction function. An ideal animal model should be long-lived and free from the systemic aging-related diseases, while maintaining other reproductive changes that emulate those in aging men. Rodent models have become the predominant species for determining the cellular and molecular changes that occur in the testis and epididymis with aging.

From mouse models we have learned that advanced age is associated with a quantitative reduction in spermatozoa, an increased number of vacuoles in Sertoli and germ cells, a thinning of the seminiferous epithelium, a reduction in the numbers of spermatocytes and spermatids, and an increase in germ cell mutations. An increase in reactive oxygen species (ROS) occurs with aging. Mice overexpressing catalase, an enzyme that helps reduce ROS, do not exhibit the age-dependent loss of spermatozoa, do not show aging-associated loss in testicular germ and Sertoli cells, and show reduced DNA lesions in spermatozoa.

The Brown Norway (BN) rat has become a commonly used model to study male reproductive aging since it has a long lifespan and is relatively free of age-related pathologies, including tumors and obesity. Striking age-related changes in the seminiferous tubules, Leydig cells and epididymides of these animals have been reported. The expression of several genes in the testis (in Leydig and germ cells) and in the epididymis is altered as a function of aging. Anomalies in the structure of the endoplasmic reticulum and nuclei of Sertoli cells, the niche-forming "nurse" cells that surround the germ cells and ensure their normal development (Chapter 7), are seen. In addition, large intracellular spaces are observed between Sertoli cells, rather than the normally embedded germ cells. The expression of genes and proteins associated with the formation of the blood-testis barrier declines prior to the barrier becoming "leaky" during aging. Effects of aging are also seen in hypothalamic-pituitary function. Importantly, the changes seen in testis and hypothalamic-pituitary functions in the BN rat with age reflect those reported in aging men.

Mating of male BN rats of increasing age to young females results in an increase in pre-implantation loss, a decrease in the average fetal weight, and an increase in neonatal deaths, indicating that the quality of spermatozoa decreases as BN male rats age. The basis for these age-related declines in reproductive function remains unclear; however, they may be a consequence of the effects of aging on gene expression and epigenetic marks of germ cells, increased sperm chromatin damage, and impaired epididymal functions (Chapter 18).

Impact of advanced paternal age in men on their progeny

Various studies have demonstrated that there is an age-related decline in conventional semen parameters, including semen volume, total sperm count, motility and morphology. Not surprisingly, natural fertility rates also decline as men age; conception at 1yr is 30% less for men >40yrs versus those <30yrs. Further, natural conceptions with men >35yrs are 1.26 times more likely to miscarry than those with men <35yrs. Pregnancies sired by fathers >45yrs showed an increased risk of late stillbirth, low birth weight and preterm birth.

Advanced paternal age and assisted reproductive outcomes

Although there are some conflicting data, overwhelming evidence indicates that advanced paternal age is associated with various adverse outcomes with assisted reproductive technologies (ARTs), including poor embryo quality, increased miscarriage rates, reduced fertilization, and decreased implantation, pregnancy, and live birth rates. One mechanism that has been proposed to contribute to the adverse reproductive outcomes in natural and assisted reproduction is impaired sperm chromatin integrity and increased DNA fragmentation rates. Indeed, the majority of studies have demonstrated an association of advanced paternal age with significant increase in DNA fragmentation.

Perinatal health

Advanced paternal age increases the risk of premature birth, gestational diabetes and newborn seizures. The odds ratio of birth defects significantly increases with each year of paternal age after adjustment for multiple confounders. These defects include cleft lip, diaphragmatic hernia, right ventricular outflow tract obstruction, and pulmonary stenosis.

Malignancies

The incidence of several cancers in progeny increases with advanced paternal age. For example, men >35yrs have a higher risk of having offspring who develop hematologic cancers compared with those whose fathers are <25yr. The risk of childhood acute lymphoblastic leukemia increases by 13% for every 5 years increase in paternal age. Other offspring malignancies associated with advanced paternal age include central nervous system tumors and breast cancer. One proposed mechanism for increased cancer risk with advanced paternal age is the telomere lengthening found in sperm as men age. Leukocyte telomeres are lengthened in the offspring of older fathers by 0.5 -2 times per year of paternal age [63-65]. While this may confer some health and longevity advantage, a higher risk for malignancy has been noted.

Mental health

Advanced paternal age is also linked to psychological and neurodevelopmental disorders in offspring. The relative risk of offspring diagnosed with schizophrenia increases progressively with paternal age from 34 years; this increased risk cannot be accounted for by other factors, such as family history of psychosis, maternal age,

parental education and social ability, family social integration, social class, birth order, birth weight or birth complications. Additionally, there is a greater risk of obsessive-compulsive disorder in offspring with advanced paternal age. Using paternal sibling comparisons, a 24-fold increase in bipolar disorder was noted in offspring born to fathers 20-24yrs versus those aged 45yrs or older. Offspring from men aged >40yrs were more than fivefold more likely to develop autism spectrum disorders compared to offspring of younger men.

Genetic disorders

An increase in several genetic diseases that occur with a low frequency in the general population is associated with advanced paternal age. These include Apert, Crouzon and Pfeiffer syndromes, achondroplasia and other conditions. Many of these disorders follow an autosomal dominant pattern, consistent with the opinion that these are mainly de novo mutations in the germline and are associated with severely debilitating phenotypes. Hence, prospective parents with advanced paternal age concerns should be informed and counselled for such risks.

Approximately 0.33% of infants are born with an altered number of chromosomes. Aneuploidies derive mainly from non-disjunction events during meiotic divisions and represent the most common heritable chromosomal anomaly. Though most constitutional aneuploidies originate in the female germline, all men produce approximately 3-5% of aneuploidy sperm; these include non-disjunction events, particularly in sex chromosomes, and are more likely to occur with aging. Most de novo structural chromosomal abnormalities are of paternal origin. Results from studies on the association of advanced paternal age and increased risks of offspring aneuploidies with structural chromosome anomalies are inconsistent. This is, in part, related to the fact that the vast majority of chromosome aneuploidies are not compatible with fetal development, leading to implantation failure or early miscarriage. Structural chromosomal rearrangements that are balanced are usually phenotypically normal and are thus undetected during childhood, while the vast majority of those that are unbalanced are not compatible with fetal development.

Mechanisms on advanced paternal age impact

Studies in animal models suggest that the constitution of the male germline is relatively robust, with far fewer spontaneous mutations compared to somatic tissues. This high level of genetic fidelity in part explains why even when men are exposed to chemotoxic agents or radiation, there is no dramatic increase in the incidence of birth defects, sperm DNA chromatin abnormalities or de novo germline mutations in their offspring. In contrast, paternal aging has been shown to be unique for the creation of de novo mutations in the male germline. Several mechanisms of age-induced de novo germline mutations have been proposed. Cumulative replication error from repeated cell divisions represents a significant source of germline mutation. Based on whole-genome sequencing studies of parent-offspring trios, approximately one to three de novo mutations are introduced to the germline mutational load of the offspring for each additional year in the father's age at conception. Selfish spermatogonial selection from preferentially amplified mitotic clonal expansion of mutated spermatogonial stem cells is another proposed theory to explain why several genetic diseases associated with advanced paternal age follow the autosomal dominant pattern. Age-related epigenomic modifications in men are speculated to increase the risk of some rare epigenetic disorders in offspring conceived with ARTs. Other proposed mechanisms involve post meiotic damage of sperm DNA secondary to the combined effects of increased ROS damaging chromatin and aberrant or inadequate repair of such damage by oocytes.

Looking forward

Though controversies exist, a preponderance of evidence from recent scientific literature affirms a negative impact of advanced paternal age on reproductive health. The first step to minimize or mitigate the negative impact of advanced paternal age is to comprehend the collective body of scientific evidence. The next step is to promote appropriate counselling to couples where the male partner is older than 40 years of age. There should be dialogues among investigators, healthcare providers, health policy makers and patients that focus on emerging data and their implications at the personal as well as societal levels.

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Chapter 54
Should there be hormone replacement therapy for aging men?

Austin Kinley and Mohit Khera

In contrast to the abrupt decline in hormone concentrations experienced by females during menopause, male hormone levels, on average, decline gradually and progressively as men age. However it is important to note that aging alone does not cause a significant decline in serum testosterone levels over time. The decline in testosterone over time is due mainly to the development of co-morbid conditions, such as diabetes, obesity, and metabolic syndrome. On average, several anabolic hormones, including testosterone, dehydroepiandrosterone (DHEA) and growth hormone (GH), experience physiologic decreases in serum concentration in older men when compared to younger men. In young adults, deficiencies in these hormones can lead to symptoms such as lethargy, decreased libido, changes in mood, and erectile dysfunction. Additionally, when younger men with documented hormone deficiencies are treated with hormone replacement therapy, there is frequent resolution of these symptoms as hormone levels normalize. Therefore, this raises the question of whether hormone replacement therapy in older men may alleviate or resolve some of the changes seen in body composition, mood, cognition, sexual function, and bone density as men age. Unfortunately, there is still limited knowledge regarding the complete benefits of hormone replacement in older men and the observed effects of therapy are frequently modest. Many of the symptoms of aging may be related to other co-morbidities or drug effects that are unrelated to the observed changes in serum hormone concentrations. In addition, there are potential adverse effects observed often with hormone replacement therapy which must be considered as well.

Testosterone

Beginning at approximately 40 years of age, testosterone levels in men begin to decline at annual rates of 1.0% for total testosterone

and 1.2% for free testosterone. This results in the mean plasma testosterone level of men in their seventh decade being 35% lower than that of younger men. There is also a corresponding observed increase in the concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and sex hormone binding globulin (SHBG), with the increased SHBG concentration accounting for the greater rate of decline of free testosterone compared to total testosterone. However, although there is an overall average decrease in serum testosterone levels as men age, it is important to recognize that these levels may never fall below the lower limit of normal for many men. The prevalence of low testosterone in elderly men is typically cited to be between 10% - 25%.

The most common presenting symptoms of testosterone deficiency include sexual dysfunction, primarily decreased libido and erectile dysfunction, alongside changes in energy level and mood. While bothersome for patients, these symptoms are not themselves life threatening. However, it is prudent to identify testosterone deficiency in older men and not dismiss complaints as common symptoms of aging, given the correlations observed between prolonged testosterone deficiency and several more life-threatening issues. Testosterone deficiency has been positively associated across numerous studies with the development of adverse metabolic outcomes, including obesity, metabolic syndrome, and type 2 diabetes, as well as coronary artery disease, osteoporosis and decreased cognitive abilities (Chapter 64).

Testosterone deficiency is associated with decreased bone mineral density in men of any age. Several studies have documented the ability of testosterone replacement therapy (TRT) to improve bone mineral density in younger men (<50 years). However, recent randomized control trials and subsequent meta-analysis showed that TRT in men above the age of 60 years with low testosterone did not significantly reverse the tendency towards decreasing bone mineral density. While there is currently no evidence to support that TRT decreases the incidence of fracture among older men, there are numerous studies demonstrating that bone mineral density increases in men on TRT.

Testosterone is also believed to influence body composition and functional status. Over the past decade, many randomized control trials and meta-analyses have investigated the effects of TRT on muscle mass and physical functioning in elderly men over a short period (< 2 years). There was some disagreement amongst the studies, but most reported that TRT produced no significant change in

muscle strength with only a modest positive effect on physical function. Studies in which TRT was administered over a longer period (≥ 3 years) do report statistically significant increases in muscle mass and strength with TRT compared to placebo; however, the overall changes were modest. When compared against resistance weight training in elderly men, TRT has not shown to be superior although it may augment the progress made when TRT is combined with resistance training.

The complete role of testosterone on cognitive function in aging men has yet to be fully understood, but it has long been hypothesized that low testosterone levels may precipitate declines in or worsen cognitive functioning in the elderly. In a recent multi-center randomized clinical trial studying the effect of TRT in hypogonadal elderly men with established age associated memory impairment, there was no improvement in memory or other tests of cognitive function over a one-year period when compared to baseline testing or placebo. The results of this trial are consistent with other smaller trials that have investigated the potential benefits of TRT in elderly patients already showing evidence of age-related memory loss or cognitive impairment. Although there is some historic data from small, short-term studies that suggests that TRT in hypogonadal elderly men with no current evidence of cognitive decline may experience slight improvement in memory and cognitive function testing, more recent studies have not reported significant changes from baseline. Additionally, there is currently no evidence that serum testosterone levels accurately predict the development of Alzheimer's or other forms of age-related cognitive impairment.

Younger men with testosterone deficiency frequently report symptoms of depressed mood and irritability that quickly correct with TRT. The same effect has been observed in older men, with a recent, large randomized clinical trial reporting statistically significant, although mild, improvements in mood and alleviation of depressive symptoms for men treated with TRT compared to placebo. Importantly, the population enrolled in this study did not include a diagnosis of major depressive disorder (MDD) in the inclusion or exclusion criteria. Several studies focusing on testosterone therapy specifically in hypogonadal men with MDD have not demonstrated resolution of depressive symptoms when compared to placebo.

Although hypogonadism is a common isolated cause of erectile dysfunction among younger men, it is infrequently the sole contributing factor to impaired erectile function in older men. As such,

younger hypogonadal men receiving testosterone replacement therapy (TRT) show greater improvement in erectile function as compared to older men. However, TRT may still be an effective treatment for older men who demonstrate particularly low testosterone concentrations or when used as an adjunct to PDE-5 inhibitors. The 2018 American Urologic Association (AUA) ED Guidelines do not recommend TRT as monotherapy for the treatment of ED. However, TRT is recommended in conjunction with phosphodiesterase type 5 inhibitors (PDE5i) in hypogonadal men because PDE5i tend to be more effective in eugonadal men. Furthermore, TRT significantly increases libido in men of all ages, and as such may be an appropriate therapy for older men reporting impairment in erectile function alongside decreased sexual desire.

Low testosterone levels in older men have long been thought to increase the risk of coronary artery disease and worsen outcomes in patients with heart failure. In fact, the 2018 AUA Testosterone Guidelines state that clinicians should counsel patients with testosterone deficiency that low testosterone levels is a risk factor for cardiovascular disease. However, the guidelines go on to state that prior to initiating treatment, clinicians should counsel patients that it is not known with certainty whether TRT increases or decreases the risk of cardiovascular events. The Testosterone Replacement Therapy (TRT) on the Incidence of Major Adverse Cardiovascular Events (MACE) and Efficacy Measures in Hypogonadal Men (TRAVERSE) Trial is set to be completed in June of 2022. This trial is the largest randomized placebo controlled TRT trial assessing time to major adverse cardiac event, which includes nonfatal MI, nonfatal stroke or death due to cardiovascular causes. It is anticipated that this trial will shed further light on the true relationship between TRT and cardiovascular risk.

There are many potential benefits of TRT in aging men, but the side effect profile of testosterone replacement should always be kept in mind. Common side effects of TRT include fluid retention, gynecomastia, polycythemia, and potentially exacerbation of sleep apnea. For these reasons, all men receiving TRT should be closely monitored by the prescribing physician throughout the duration of treatment.

In an older male patient with symptoms consistent with hypoandrogenism but with normal testosterone levels on laboratory testing, it is prudent to search for other causes of the patient's symptoms. Many other pathologies or drug side effects may cause many of the symptoms associated with low testosterone and these

etiologies should be further explored. The formulations of testosterone approved by the Food and Drug Administration (USA) as of 2021 can be seen in Table 1, Chapter 52).

DHEA

Levels of DHEA typically peak in the third decade of life, after which there is a gradual decline in serum concentration. By the time men are between 70-80 years of age, DHEA concentrations are approximately 20% of what they were at their peak. As a result, DHEA is frequently marketed as an over-the-counter supplement to prevent symptoms of aging. However, currently available data limits the understanding regarding both the potential benefits and harms of DHEA supplementation in older men.

Available data does suggest that DHEA causes statistically significant improvement in bone density in older men compared to placebo; however, the improvements are mild and much less than is achieved with other currently available treatments for osteoporosis. In regard to cognitive functioning and mood, small studies have produced conflicting data. Although some studies did report some slight improvement in episodic memory and mild improvements in mood, just as many studies report no significant changes within these domains. Additionally, although DHEA is converted *in vivo* to other more potent androgens, supplementation has not shown any statistically significant improvements in libido or erectile dysfunction among older men.

There are currently no studies detailing adverse outcomes of DHEA supplementation. Although several sources discuss a theoretical risk of progression of hormone sensitive cancers (e.g., prostate and breast) or increased prostate volume, there is no data supporting these hypotheses currently available in the literature. At present, there is insufficient data to recommend routine DHEA supplementation to prevent the physiologic changes related to aging in men. Although there are no known adverse effects of supplementation, any potential benefits are thought to be insignificant.

Growth Hormone

Similar to testosterone and DHEA, growth hormone and IGF-1 levels gradually decline as men age. However, it is currently illegal in the United States to prescribe growth hormone as an anti-aging treatment. Given the known influence of growth hormone in younger

populations on body composition and metabolic function, it has been theorized that supplementation in older adults may alleviate symptoms of aging. However, currently available studies have failed to demonstrate significant clinical benefit. A handful of clinical trials have shown a mild increase in lean body mass with a corresponding decrease in body fat; however, no other improvements in metabolic function or body composition have been noted.

Additionally, there are known adverse effects of growth hormone supplementation that have limited further study. Frequently observed side effects include carpel tunnel syndrome, insulin resistance, edema and arthralgias. There are to date no studies detailing cancer risk in older patients receiving growth hormone supplementation.

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Chapter 55
What is the place of psychosocial issues in andrology?

William Petok

Andrological problems can take many forms and include a range of problems such as the impact of environmental toxins on reproduction, impaired erectile function, and testicular cancers. While it is essential to consider the specific systems affected and how to treat those impacts, it is equally important to remember that each of those problems is taking place within a man whose life includes relationships with others. These relationships will vary on their level of intimacy, both psychologically and physically. As a result, attention to how his diagnosis and treatment could affect those relationships is critical when comprehensive care is the goal.

As a rule of thumb, most men know less about their health in general than women. And men have less sexual and reproductive health information, primarily because they typically seek care for these issues when there are problems as compared to women who generally receive at a minimum reproductive health care from the onset of puberty. While there is great variability in how sexual and reproductive health information is delivered in educational settings, it is safe to say that it frequently focuses on how not to create a pregnancy or contract a disease, limiting knowledge about fertility as well as the broader aspects of sexual health that include pleasure. Men are also less likely to ask for help in general and the notion of psychological "help" can be a deterrent to men actually participating in psychotherapeutic activities. Finally, the stigma attached to reproductive and sexual health issues can prevent men from discussing them with their partners.

Psychosocial issues resulting from andrological problems can be wide ranging. Most commonly, they present as increased anxiety, stress, depression, and a sense of loss of control. Guilt and shame can also be associated with them. Some men will turn to activities they think will reduce these uncomfortable feelings such as substance abuse, gambling or increased risk taking in other forms. The focus here will be on the former, but a competent practitioner will

consider maladaptive behavior as a potential consequence of an andrological diagnosis and have referral sources available for patients in need of them.

Sexual problems and their treatment

Erectile difficulties and ejaculatory control are very common presenting problems in the andrologist's office. To a lesser degree patients with low sexual desire may request help. A first line intervention is often information about normal sexual functioning that many men may not have.

Recent epidemiological studies suggest that erectile dysfunction (ED) for all age groups internationally ranges between 32% and 80%, with higher numbers occurring with increased age. Other health conditions can be at play, including diabetes, cardiovascular disease and substance or alcohol abuse. In addition, many pharmaceutical interventions for these disorders can impact erectile function. For example, SSRIs (selective serotonin reuptake inhibitors), diuretics, and beta-blockers all have known adverse impact on erections.

It is important for men to know that erections normally wax and wane in any one sexual encounter and are never as described in literature or as seen in pornographic representations, always rigid and long lasting. All too frequently, a man experiences a brief loss of erection and internally translates it into chronic ED. This performance anxiety associated with his internal dialogue can contribute to persistence of the problem. Typically, the man will worry about what will happen the next time he attempts sexual activity rather than focus on arousal and pleasure during the encounter. Psychotherapeutic interventions for ED predate the use of PDE5 inhibitors and can be most useful when the problem is clearly psychogenic in origin and are useful even when there is a physiological component. A sex therapist will collaborate with the man and eventually bring his partner into the treatment, if he has a regular one. Therapy generally consists of both education about what is normal, substitution of more helpful thoughts, and specific exercises to help the man become more confident about his erection capacity. For an older man with medical complications or the normal impact of aging on erections, helping shift from intercourse as his primary sexual activity to other forms of pleasure may be the goal.

Premature/rapid ejaculation (PR/RE) has an estimated incidence rate of 20-30% across all age groups and is a frequent presenting

problem among younger, less sexually experienced men. It is a complicated disorder because there is no universally accepted definition. Premature or too rapid for what? One man's "rapid" is more than acceptable to another man or his partner. Ejaculatory latency time, control over ejaculation and satisfaction with sexual intercourse have all been utilized as descriptors for the disorder. PE/RE can cause distress, anxiety, depression, shame, reduced libido, impaired interpersonal relationships, anxiety about intercourse and the avoidance of sexual relationships. It may be useful to conceptualize the issue, particularly with younger men, as a learning process. That is, with experience a man learns more about his sexual response to various forms of stimulation and learns to moderate his activity to receive and provide increased pleasure to himself and a partner.

As with ED, PE/RE may occur in conjunction with other medical conditions such as diabetes, hypertension, hyperthyroidism, alcoholism, or the use of recreational drugs. Depression stress, or anxiety about sexual performance can also function as triggers.

The concept of "ejaculatory inevitability" or that point in the sexual response cycle when there is no preventing an ejaculation can be useful for talking with men. Behavioral interventions help him appreciate when he is reaching that point, reduce anxiety about the problem, and help him communicate better with a partner during sexual activity. The "stop-start" technique, extended foreplay and alternate positions for intercourse are other interventions employed for the disorder. More recently the use of topical medications and SSRIs (e.g. dapoxetine) for their adverse side effects of delaying ejaculation have been recommended. Psychotherapy combined with the use of medical treatment is often recommended due to the impact of the disorder on the man's emotional response and the corresponding impact on his relationship with his partner.

For both ED and PE/RE, Cognitive Behavior Therapy (CBT) is a treatment of choice. As noted earlier, helping the patient change his cognitions about the problem as well as his behavior can be essential for long term success. The presumption is that anxious or distorted thoughts will help maintain the sexual problem. Common distortions include "if I was unable to get erect last night, I won't tonight," "I know she thinks I'm inept sexually and a failure as a man," "I'm not a real man because I can't control my orgasm," "If I can't get this right, she'll leave me for someone else." Prior research confirms that counseling in conjunction with medical interventions helps maintain improvements in sexual function as well as relationship quality.

Infertility

Infertility affects an estimated 15% of the population, in close to equal amounts for men and women, considering instances where combined male and female factor coexist. Men do have emotional reactions to infertility, both when the primary diagnosis is with their partner and when a male factor is diagnosed. And community concepts of masculinity often include paternity. Thus, a man who is unable to produce children can view himself as "less than a man." Traditionally, men learn to cope with sadness by hiding feelings. Stereotypes for maleness include toughness and a lack of emotionality. Denial and avoidance of the topic are common coping strategies for men with a male factor diagnosis. This can put them in conflict with their female partners who may interpret this response as not caring. At the same time, men receive messages about being strong for partners which often translates into not disclosing how they themselves feel about the fertility challenge. As a result, she may think he does not care. The disruption that can occur to a relationship is obvious.

Sometimes infertility and sexual prowess are inappropriately conflated. Or a provider may refer to his infertility as "shooting blanks" or some other emasculating phrase, increasing a sense of stigmatization. This has the potential to cause significant personal and interpersonal distress for the infertile man and his partner.

An infertility diagnosis may also lead to sexual problems that include ED or a lack of desire. Conversely, sexual difficulties can also have an impact on fertility. ED that prevents penetration, PE/RE that leads to extravaginal ejaculation and male orgasmic disorder in which intravaginal ejaculation does not take place can all be causes of infertility.

Counseling for the emotional impact of infertility is often recommended to couples in medical treatment. Since the focus of much fertility treatment occurs within OB/GYN and reproductive endocrinology offices with women as the focal point of treatment, men are often an afterthought with regard to their psychological reaction. Men are less likely to seek counseling services in general unless they know someone who has previously had a positive experience with it. Not surprisingly, men tend to focus more on discussion of activities than emotions. As a result, referrals for counseling may go unheeded because the man presumes he will have to engage in stereotypic female behavior. That is, he will be required to talk about emotions, particularly negative emotions like sadness and grief.

The availability of a male counselor and the use of language that is masculine in making the referral ("this may help you tackle the problem" vs. "it will help you cope with your grief") have been shown to increase men's usage of these resources. Label counseling sessions as meetings, conversations, or consultations as opposed to counseling or therapy. Another promising development in this area is the increasing availability of online support networks for men only. Men are notoriously unlikely to attend infertility focused in-person support groups but have reported satisfaction with a system that allows them to be anonymous and discuss emotional issues at the same time with other individuals like themselves who are experiencing similar situations. This can be particularly helpful for men who may be considering the use of donor sperm for azoospermia and are concerned about bonding with a child who is not from their genetic line. A trained infertility counselor can offer good advice on what constitutes parenting and how donor conceived children bond with parents. A group may provide him with an additional outlet for his concerns and could include men who have successfully made the decision to go forward with using donor sperm.

Other problems

Andrological related cancers present a unique set of problems for the treating physician. Most men will be concerned about the impact of treatment on their sexual and reproductive functions but may be reluctant to ask or be overwhelmed by the impact of a diagnosis. A thoughtful explanation of how the cancer and its treatment will likely affect both sexual and reproductive function is advisable. The physician should initiate this conversation and not wait for the patient to bring it up. Addressing their concerns about aging and a discussion on reasonable expectations for how sexual function normally changes are also useful interventions.

Additional thoughts

Men are comfortable with statements such as ''many men experience concerns about their low sperm count and what it means about their ability to have a family. Have you experienced similar thoughts or feelings?'' Similar normalizing statements about sexual problems are also helpful. This type of statement-question technique is an effective way to initiate a dialogue on a sensitive topic, normalizing it at the

same time. With men, who typically do not seek support but clearly benefit from it, normalizing the idea of supportive consultation seems to be critical to improve its utilization.

Medical problems that compromise sexual and reproductive function can be difficult to discuss for most people. Men are at a decided disadvantage in this arena and careful attention to the psychosocial impact of andrological problems can reduce the stress induced by them. To repeat, it is important to remember that each of those problems is taking place within a man whose life includes relationships with others. Taking the entire system into account and offering appropriate resources is crucial to your patient's overall health.

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Chapter 56
What is benign prostatic hyperplasia and how is it treated?

Manuel Belmonte Chico Goerne, Abdulghani Khogeer, Melanie Aubé-Peterkin and Serge Carrier

Prostate anatomy

The prostate, a male reproductive gland, originates from the endodermal urogenital sinus. It has an ovoidal shape; in adults, it measures 4x3x2 cm approximately, and weights around 20 gr. It's located anterior to the rectum, above the urogenital diaphragm, posterior to the symphysis pubis, and below the base of the bladder (Chapter 20). It surrounds the urethra, which travels through the gland from the bladder neck to prostate's apex. Based on the antomo-histological characteristics, it can be divided into three zones, the peripheral zone, as the largest (70% of the gland), the central zone (20%), which is surrounded by the peripheral zone, the transitional zone (10%), where the benign prostatic hyperplasia (BPH) most commonly develops and lastly, the anterior fibromuscular stroma. Close to prostate apex, the ejaculatory ducts (which travel across the gland) are located. The ducts are the result of the fusion of seminal vesicles and vas deferens, and they empty the transported semen into the urethra at the level of seminal colliculus (verumontanum).

Prostate physiology

The gland physically participates on urinary bladder output and urethral lumen control. By closing the urethra, it controls seminal fluid transmission and impedes semen from entering the bladder during ejaculation. During urination, the central zone muscles prevent urinary influx into ejaculatory system by closing the prostatic ducts. As an exocrine gland, it secretes 20-30% of seminal fluid volume (as alkaline fluid). The components of this fluid are PSA, prostatic acid phosphatase, citric acid, zinc, spermine, and prostatic inhibin. These secretions liquefy semen and enhance sperm viability by stimulating sperm motility and reducing urethral acidity. PSA, an

androgen-regulated serine protease, it's used to help in the early diagnosis of prostate cancer (PCa), and to assess progression and treatment outcomes on patients with the diagnosis. Finally, 5α-reductase type 2 convert testosterone to dihydrotestosterone in the prostate.

Prostate physiopathology

The prostate is susceptible to various benign and malignant diseases. The 3 most common conditions affecting the prostate are prostatitis, benign prostatic hyperplasia, and prostate cancer. Prostatitis is an infectious or non-infectious inflammation of the gland, which may present either acutely or chronically, and its main symptom besides low urinary tract symptoms (LUTS) is pelvic pain. PCa was the fifth cause of cancer leading death in USA in 2020. The most common clinical presentation is asymptomatic prostate-specific antigen (PSA) elevation. Treatment depends on the disease stage ranging from active surveillance, surgical or non-surgical treatments or combination of both. Of note, PSA elevation may also be related to BPH and prostatitis, as well as recent manipulation.

What is BPH?

BPH is a non-malignant prostatic glandular enlargement (Fig. 1). There is no global accepted epidemiological definition of BPH. However, disease prevalence increases with age and affects approximately 50% of men over 50 years and up to 80% by the age of 80. Histologically, it's an increase in epithelial and, mainly, stromal cell numbers (hyperplasia). This increase in cell numbers, is secondary to proliferation of both, epithelial and stromal cells, the imbalance between cell renewal and death (impaired cellular apoptosis), or a combination of both. Some suspected contributing factors are aging, family history, hormonal, and growth factors, as well as inflammatory factors. The pathophysiology is complex, hyperplasic prostatic tissue increase urethral resistance, causing outflow obstruction with subsequent compensatory alteration in the urinary bladder function, which is reflected clinically as LUTS.

Symptoms and diagnosis

Despite not all men with BPH present symptoms, of all the conditions leading men to develop LUTS, it's the most frequent, and the one most studied so far. The symptoms and its severity can vary

widely among patients and non-prostatic causes must be excluded. LUTS attributed to BPH include storage and/or voiding symptoms (Table 1). Some patients can develop recurrent UTI's, bladder stones, impaired kidney function, hematuria, and urinary retention. The diagnosis is done clinically and can be supported with further exams. Evaluating a patient with LUTS mandates initially, a full medical history, assessment of symptom severity with a validated questionnaire, physical examination including a digital rectal exam (DRE), and urine analysis (to rule out any other LUTS cause). Serum creatinine, PSA, urine cytology, post-void residual urine volume (PVR), flow rate (Qmax), urodynamics, obstructive sleep apnea assessment, and ultrasonography are supplementary optional tests suggested in selected cases. The International Prostate Symptom Score (IPSS) is a questionnaire that quantifies symptom severity and frequency using 7 questions; the classification ranges from mild (0-7) to moderate (8-19) or severe (20-35) and includes a final question regarding Quality of life (QoL). DRE provides an approximation of prostate size, and mainly, it's done to detect nodules that may indicate PCa. Prostate size doesn't correlate with symptom severity. PSA is recommended for those patients in whom its measurement may change the management of their voiding symptom or those with 10-year life expectancy in whom the presence of PCa would divert the management; PSA can provide size estimate and prediction of progression risk. PVR and Qmax are suggested for men considering surgical therapy.

Storage:
Frequency
Nocturia
Urgency
Incontinence

Voiding:
Slow stream
Splitting or spraying
Intermittent stream
Hesitancy
Straining

Post-micturition:
Feeling of incomplete emptying
Postmicturition dribble

Table 1. Classification of lower urinary tract symptoms (LUTS)

Unless clinically indicated, it is not recommended to perform in the initial evaluation urethro-cystoscopy, urodynamics, upper or lower urinary tract imaging or prostatic biopsy.

Treatments

Symptom severity and impact on QoL, will determine whether treatment is necessary or not. Treatment should be offered to patients with moderate and severe symptoms and for those with severe bother regardless of the symptom severity.

For patients with mild and/or minimally bothersome symptoms, lifestyle changes are suggested, including fluid restriction (particularly in the evening), avoidance and monitoring of certain drugs, timed voiding avoidance or treatment of constipation and pelvic floor exercises.

Alpha blockers, and/or 5-alpha-reductase inhibitors (5ARIs) are the mainstay of medical treatment options; however, antimuscarinics, beta-3 agonists and PDE5 inhibitors (PDE5-I) can also improve LUTS.

Surgery is indicated in men with refractory or recurrent urinary retention, recurrent urinary infections, bladder stones, recurrent

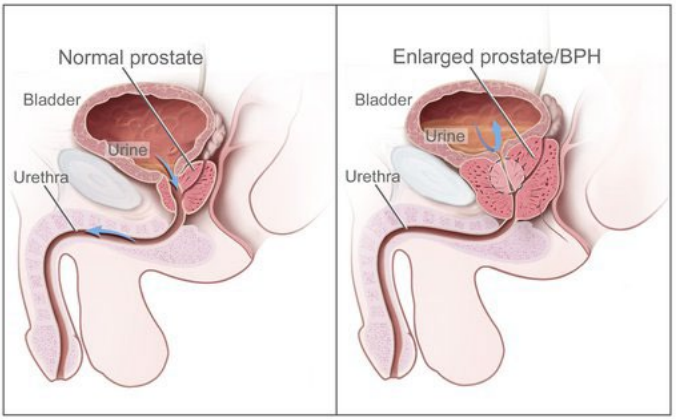

Figure 1. Two-panel drawing shows normal male reproductive and urinary anatomy and benign prostatic hyperplasia (BPH). Panel on the left shows the normal prostate and flow of urine from the bladder through the urethra. Panel on the right shows an enlarged prostate pressing on the bladder and urethra, blocking the flow of urine.
Source: National Cancer Institute.

hematuria, renal insufficiency secondary to BPH, LUTS refractory to other therapies, according to patient preference.

Oral medication

Alpha-blockers are an excellent first-line options and offer dynamic relaxation of the prostatic urethra to improve urine flow. The choice of which agent to prescribe is based on patient comorbidities, age, and possible side effects. These medications don't alter BPH natural progression. The most common side effects are dizziness and retrograde ejaculation.

5-ARIs shrink the prostate volume approximately 20-30 %, altering natural BPH history, and decreasing the risk of acute urinary retention. These medications are recommended when the prostate volume is >30cc and/or PSA > 1.5 ng/dL. Side effects predominantly sexual in nature (e.g., erectile dysfunction, and decreased libido). Gynecomastia is an uncommon side effect.

Alpha-blockers and 5-ARIs used in a combined fashion have shown superiority in improving IPSS and peak urinary flow (Qmax) compared to monotherapy.

Antimuscarinic and beta-3 agonist notably improve storage symptoms. Precautions regarding urinary retention should be taken, especially in elderly patients and those with PVR >200cc or with significant BOO.

For patients with LUTS and erectile dysfunction (ED), Tadalafil (a PDE5-I) taken on a daily dose of 5mg has proved to improve both.

Transurethral resection of the prostate (TURP)

Using either monopolar or bipolar energy, TURP is the surgical procedure of reference in patients with 30-80 cc prostates. Complications are most common in larger prostate volumes (>60 cc) and include extravasation, bleeding, infection, persistent or recurrent retention, need for repeat surgery, incontinence, bladder neck contracture, ED, and TUR syndrome. Morbidity is decreased with bipolar TURP, especially when it comes to bleeding and TUR syndrome.

Photoselective Vaporization of the prostate (PVP)

Potassium-titanyl-phosphate (KTP) or Greenlight laser, is the most used energy to perform PVP. Functional outcomes are similar to TURP with regard to IPSS reduction and Qmax improvement, and PVP is a treatment of choice for patients actively taking anticoagulant or anti platelet medications due to decreased risk of bleeding. Retreatment

rates are low on <80 cc prostates. The main drawbacks are transient post-operative dysuria, higher re-intervention rates for larger prostates, and lack of pathological specimen.

Open Simple Prostatectomy (OSP)

OSP is effective for patients with >80 cc prostates and is the treatment of choice for large glands when LEP is not available. Indications also include concurrent bladder procedure (e.g., diverticulectomy or cystolithotomy), and hip disease precluding the dorsal lithotomy position. OSP is the most invasive surgery for BPH, and requires a longer hospitalization stay and catheterization. Post-operative complications also include transient urinary incontinence (8-10%), and the transfusion rates are higher than with any other technique.

Laser Enucleation of the Prostate (LEP)

LEP mimics an open prostatectomy, but performed in a minimally invasive fashion, using an energy (such as laser), to enucleate the adenoma trans-urethrally. A variety of sources may be used, including holmium, thulium, and KTP lasers (HoLEP, ThuLEP, and GreenLEP respectively), as well as bipolar energy (BipoLEp). Enucleation is a size-independent technique which removes more tissue than any other technique. After HoLEP, all subjectives (IPSS and QoL) and objectives (PVR and Qmax) parameters improve immediately, and the PSA decreases by 75%. Reoperation rates are <1.5% at 18 years. Complications are the same as with TURP, without the risk of TUR syndrome, and with reduced risk of bleeding, and shorter catheterization times. Transient urinary incontinence is the most common early post-operative event and has a 4.3% chance of being permanent. ThuLEP have shown similar enucleated weight, operative time, hospital stay and catheterization time, with no significant differences in PSA, Qmax, PVR, IPSS and QoL.

Minimally Invasive Surgery Techniques (MIST)

These technologies were developed to limit surgical morbidity, decrease anesthesia risks, as well as post-operative recovery period.

Prostatic urethral lift (Urolift™)

It's an option for patients who desire preservation of erectile and ejaculatory function and who have a prostate volume between 30-80 cc. Having a median lobe is no longer considered as a contra-indication, as the middle lobe can be tacked to the side. The procedure is performed under cystoscopic guidance and can be

done under local anesthesia. The delivery device (a disposable cartridge with nonabsorbable monofilament suture tabbed implants) is placed through the urethra, then the implants are placed compressing the lateral lobes, creating an open prostatic urethra. Subjective parameters improvement has been shown by cohort studies, meanwhile, Qmax and PVR have only small improvement. Side effects are dysuria, hematuria, discomfort, and recurrence.

Prostatic Artery Embolisation (PAE)

PAE, performed by interventional radiologists, is a procedure during which, the prostatic arteries are blocked with particles, causing ischemia of intraprostatic vessels and consequently progressive shrinkage of the prostate. Indications include patients not suitable for surgery or anesthesia, men who want to preserve ejaculation, >65 cc prostate, and recurrent hematuria. The failure rate is 19% and 15% of the patients require a TURP within the first year after PAE. Complications include dysuria, hematuria, hematospermia, urinary retention non-target embolization and recurrent treatment.

Water Vapor Therapy (Rezum™)

This system transforms sterile water into vapor steam (thermal energy), which disrupts the cell membranes of the prostatic transition zone causing cell necrosis, and subsequent tissue ablation. This therapy is recommended for men with prostates <80 cc who are interested in preserving ejaculatory function. The procedure is usually performed as day surgery, using local anesthesia and/or sedation. Presence of median lobe obstruction is not an exclusion criteria. 5-year data have shown that IPSS, Qmax, QoL, and PVR improve significantly after Rezum™, and sexual function is not affected.

Aquablation

It consists of image-guided robot-assisted waterjet ablation. Guided by transrectal ultrasound, hydrodissection is performed using high-pressure saline, ablating the prostatic parenchyma, but sparing its capsule and vessels. Despite demonstrable efficacy in >150 cc prostates, in outcomes are comparable to TURP in <80cc prostates. Sexual function is preserved in almost 100% of the cases, and retreatment rate at 5 years is low.

Summary

BPH is a frequent condition that can lead to male LUTS. Men presenting with urinary symptoms should be fully evaluated to assess the severity of these symptoms and the impact on their QoL. Once evaluation is complete, therapeutic shared decision-making is guided by, symptom severity, patient age, comorbidities, expectations, and potential treatment side effects to select the best treatment.

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Chapter 57
What is prostate cancer? How is it diagnosed? What is its prevalence?

Joseph Alukal

Prostate cancer is the most common solid malignancy in men, estimated to affect two million American men at present (cdc.gov); moreover, more than 200,000 men are expected to be diagnosed with prostate cancer in the year 2023 (cancer.org). The prostate is centrally important to male reproductive and sexual function and prostate care drives a significant percentage of visits to the urologist annually. Urologists, and especially andrologists, are behooved to have a thorough understanding of both the prostate itself, its function, and its capacity to become cancerous.

The growth and development of the functioning prostate depends on testosterone (T) and its metabolite dihydrotestosterone (DHT). These two hormones enable the growth and proliferation of the glandular component of the prostate through binding and activation of androgen receptor (AR) expressed by prostatic epithelial cells (Chapter 3). Research has established that the activation of this receptor enables both benign prostatic hyperplasia (BPH, Chapter 56) and carcinogenesis within prostatic epithelial cells leading to prostate cancer (PCa). Both diseases are common and burdensome conditions in aging men (Chapter 52). However, the relationship between testosterone and these two conditions is unclear; the simple observation that hypogonadism (low T), BPH, and PCa are all age-related conditions reinforces this confusion.

In this chapter, we review the existing data regarding these relationships. Pharmacologic treatments for prostate cancer depends upon manipulation of these pathways; we will review these treatments, as well as outline future directions for treatment that are being explored.

Androgen Physiology and Prostate Carcinogenesis

The data regarding the relationship between testosterone and prostate cancer are numerous and varied in terms of their implications.

However, one aspect of this complex relationship is well illustrated in the studies examining 5α-reductase (5-ar) inhibitors.

Two large, prospective, randomized, placebo controlled trials were done examining the relationship between chronic 5-ar inhibitor usage and prostate cancer incidence: The REDUCE trial and the Prostate Cancer Prevention Trial (PCPT). Both studies demonstrated an approximate 30% risk reduction in the development of prostate cancer over the ten year window of the study. Some initial concern regarding slight increases in high risk cancers in the treatment arms of both studies was dismissed initially as being most likely due to detection bias (as opposed to treatment effect); the long term follow-up of the PCPT, published in 2013, supported this theory, at least in so far as disease specific mortality in the treatment arm was far less than in the placebo arm (thereby implying no meaningful increase in high risk, clinically significant cancers with 5-ar inhibitor use).

The conclusion reached therefore, is that a) DHT levels can in part drive prostate carcinogenesis and that b) decreasing these levels, in addition to preventing prostate enlargement, can prevent prostate cancer. The corresponding question of whether T levels themselves influence prostate cancer risk remains unanswered. Numerous data exist regarding this specific question; they point to different conclusions. Some studies implicate low T levels as conferring a higher likelihood of high risk prostate cancer, implying that more than one pathway for prostate carcinogenesis might exist. The data regarding management of metastatic prostate cancer through chemical castration further supports the relationship between testosterone levels and prostate cancer progression.

Regardless, given that low T levels should correlate to low DHT levels, one would think that the observations from the above trials would hold and that hypogonadal patients would be less likely to develop any kind of prostate cancer. Instead, the common epidemiological observation that both prostate cancer and low T are diseases of aging men confounds this picture. A man in his 80s is far more likely to have both low testosterone and prostate cancer than he was in his 20s. Whether or not this observation is correlative but not causal remains to be proved. Certainly, given the common and burdensome nature of both problems, further study is warranted.

Diagnosis and Treatment of Prostate Cancer

For many years, the diagnostic algorithm for patients suspected of having prostate cancer involved PSA (prostate specific antigen) testing followed by transrectal ultrasound guided prostate biopsy in patients with PSA abnormality. There have been numerous improvements upon this algorithm; first, many of the large studies evaluating prostate size depend upon transrectal ultrasound for measurement of prostate volume. This is a highly variable modality; inexact measurements can be obtained for any number of reasons including operator variability and patient discomfort. Second, prostate cancer incidence in both the REDUCE and PCPT trials was determined using transrectal ultrasound guided prostate needle biopsy. This modality is also inexact. Numerous data show clearly that both prostate volume measurement and prostate cancer detection are improved upon with utilization of multiparametric MRI of the prostate. Follow up studies incorporating MRI as a means of following prostate volume change and development of prostate cancer might help illuminate the true effect of testosterone and DHT within the prostate. Biopsy of the prostate – whether ultrasound or MRI guided – results in a pathologic Gleason score (sum of two numbers 1-5; e.g. Gleason 3+4, with higher numbers characterizing further de-differentiation and increased aggressiveness); more recently Gleason scores have been simplified into Gleason grade groups 1-5 again with a higher number indicating a more aggressive cancer.

Better diagnostic accuracy has enabled more accurate characterization of low, intermediate, and high risk prostate cancers. This has in turn enabled some significant fraction of patients to safely embark on active surveillance of their prostate cancer (observation without definitive treatment, thereby precluding side effects associated with standard treatments). The gold standard treatments of surgery (radical prostatectomy, either open or robotic assisted) or radiation (including external beam radiation, brachytherapy, stereotactic targeted beam, and proton therapy) have been joined by focal treatments designed to treat the cancer and leave the remainder of the prostate unaffected. Modalities enabling focal treatment of prostate cancer include cryotherapy, high intensity focused ultrasound, and steam/vaportherapy. These treatment options are made possible again only by localization of prostate cancer as enabled by MRI of the prostate.

Future Directions

Assays of T and DHT represent a source of variability as well; both measurements are subject to diurnal variability – testosterone levels to a greater degree - and this introduces a further source of inaccuracy to the existing data. "Who is the truly hypogonadal patient?" is a question that first needs to be answered before figuring out whether or not he is at increased or lesser risk of prostate cancer. Assays of AR function at the cellular level including the upregulation of downstream genetic targets of activated AR could represent a future means to more accurately distinguish hypogonadal from eugonadal patients.

Lastly, data from another study published in 2015 by Finkelstein et al neatly illustrated that our understanding of hypogonadism as a disease driven only by T levels is incomplete. Patients enrolled in this study were eugonadal; they were initially treated with a gonadotropin releasing hormone receptor agonist (leuprolide) which subsequently resulted in castrate levels of testosterone. They were then given varying degrees of testosterone replacement; some were replaced to therapeutic levels, some to sub- or supra-therapeutic levels. They were also randomized to treatment with an aromatase inhibitor (anastrozole) or a placebo; blockade of aromatization in the treatment arm resulted in absent levels of estrogen in these patients, this was in spite of normal or near normal testosterone levels. Unexpectedly, some patients in the treatment arm, again with normal testosterone levels and low estrogen levels, complained of symptoms that are normally attributed to low testosterone (central obesity, fatigue, low libido). This effect could only be explained by the inadequate levels of estrogen in these patients. Previously, no data existed that implicated estrogen levels in the male in any of these processes.

The idea that testosterone, DHT, and estrogen are all powerful hormones with effects on male physiology is incompletely understood. The relationship between these three hormones within the prostate and the possibility that different patients respond differently to these hormones at the cellular level (in much the same way that estrogen and progesterone have different cellular effects in some women versus others with regard to breast cancer) warrants further investigation.

Conclusions

The relationship between testosterone and prostate health is centrally important to men's health. Prostate growth, sexual and reproductive function, the risk of prostate cancer, and the likelihood of urinary symptoms related to prostate obstruction; all of these men's health issues are in some fashion related to testosterone. While the existing data is extensive and illuminates this many faceted relationship, our understanding of the pathways by which testosterone and the prostate influence each other is incomplete. Further research is certainly warranted given the central importance of prostate health to the male population.

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Chapter 58
What are the treatments for organ-confined prostate cancer and how effective are they?

Partha P. Banerjee

Organ-confined or localized prostate cancer is a malignancy in which there is no outgrowth or extension of tumor beyond the capsule of the prostate. The prostate gland is an accessory sex organ in men which provides a component of seminal fluid during ejaculation. However, with age, 1 out of 8 men will develop tumors in the prostate gland and 1 out of 41 men will die from this disease. The adenocarcinoma develops in the peripheral region of the prostate gland. In general, prostate cancer is slow growing in the majority of the patients as it takes decades to develop; however, in some patients it eventually metastasize to various parts of the body (e.g., lymph nodes, lung, brain and bone) and becomes deadly. In this chapter we will discuss how the organ-confined/localized prostate cancer is diagnosed, what the hallmarks of organ-confined prostate cancer are, and what the current treatment options for organ-confined prostate cancer and their effectiveness are.

How to detect an organ-confined prostate cancer?

The clinical course of newly diagnosed organ-confined prostate cancer can vary. Prostate cancer is diagnosed by evaluating serum concentration PSA (prostate specific antigen), digital rectal examination (DRE), magnetic resonance imaging (MRI), computed tomography (CT) scan, bone scan and prostate biopsy.

PSA (also known as kallikrein III, seminin, semenogelase, γ-seminoprotein and P-30 antigen) is a 34-kDa secretory glycoprotein serine protease produced in all mature glandular epithelial cells of the prostate gland as well as by prostate cancer cells. In adult men, blood PSA level ranges from 0.5-4 ng/ml and its level increases with the development of prostate cancer (above 4 ng/ml to 600ng/ml or more). Using blood PSA as a screening tool is controversial due to the fact that it is also increased in benign prostatic hyperplasia

(BPH) and prostatitis; however, it is the easiest and most commonly used test we have at this point. PSA concentrations reaching between 4 to 10 ng/mL are considered suspicious. Once PSA level is suspicious, digital rectal examination of the prostate through the wall of the rectum is generally performed. Hard or nodular areas in the surface of the prostate gland indicate that cancer may be present and require further investigations.

MRI is performed to obtain a detailed picture of the prostate gland and the surrounding tissues. MRI helps the urologist decide whether the patient needs a biopsy, and which areas of the prostate should be targeted for needle biopsy. Similarly, CT scan can be done after a prostate biopsy to determine if the cancer has spread outside the prostate to the surrounding lymph nodes. To determine if the lump of tissue is malignant or not, a thin needle biopsy is used to take small pieces of tissue from the prostate. Histological assessment of the tissue is essential to determine if a malignant tumor has formed. Biopsy results also show how aggressive the cancer is (how likely it is going to spread outside the prostate gland). Under the microscope, pathologists give a Gleason score or grade based on the patterns of epithelial cells in its surrounding stroma (Fig. 1). The Gleason grade is given from 1 to 5. The Gleason score is determined by adding together two Gleason grades. The first is the most prevalent grade in the entire samples collected and the second is the highest grade within the tissue biopsy. When these two grades are added together, the total is called the Gleason score. For example, if the biopsy samples from a patient show that most of the cancer seen is grade 3, and the highest grade of any other cancer seen is grade 4, then the Gleason score will be 7 (3+4). It is also important to note that individual scores are also very important. For example a Gleason score of 4+3 dictates that the cancer is relatively more aggressive compared to a score of 3+4, as there is more grade 4 cancer, although the total Gleason score is 7 in both cases. In some patients the Gleason score could be made up of two of the same Gleason grades, such as 3+3, this means that no other Gleason grade was seen in the entire biopsy samples. A Gleason score of 6 (3+3) will be low risk compared to a Gleason score of 10 (5+5) which is a high risk. In addition to Gleason scores, Gleason 'grade group' is also a newer system for describing how aggressive a patient's prostate cancer is likely to be. A grade group (1 to 5) is given based on the

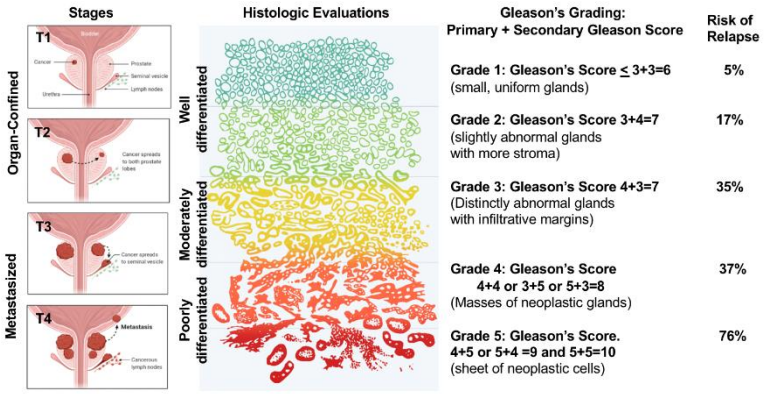

Figure 1. The cartoon showing stages of organ-confined and metastatic prostate cancer with histologic features (well-differentiated, moderately-differentiated and well-differentiated) and Gleason scores (created with BioRender).

Gleason score. A Gleason score of 6, is equivalent to grade group 1, suggests the cancer is likely to grow very slowly, if at all, for example localized prostate cancer. Whereas a Gleason score of 7, will be equivalent to a grade group 2 or 3, suggests the cancer may grow at a moderately quick rate and a Gleason score of 8, 9 or 10, will be equivalent to grade group 4 or 5, indicates the cancer may grow more quickly. In addition to the Gleason score, MRI and CT scan results can be used to determine if there is any metastasis of cancer cells and how far they has spread. This staging system is usually recorded using the TNM (Tumor-Nodes-Metastases) system. The T stage shows how far the cancer has spread in and around the prostate. The N stage shows if the cancer has spread to surrounding lymph nodes and the M stage shows if the cancer has spread to other parts of the body. T1 prostate cancer can't be felt during a DRE or scans; however, it can be detected during the evaluation of tissue biopsies. T2 prostate cancer can be detected during a DRE or can be seen on MRI or CT scan, but is still contained inside the prostate. This staging can be subdivided as T2a: when the cancer is in half of one side (lobe) of the prostate, or less; T2b when the cancer is in more than half of one of the lobes, but not in both lobes of the prostate; and T2c: dictates the cancer is in both lobes but is still inside the prostate. Higher stages (T3 and T4) indicate that the prostate cancer

has spread outside the prostate and is no longer an organ-confined prostate cancer. The N stage diagnosis means the cancer has spread to the lymph nodes near the prostate. For example, if patient's blood PSA is less than 10 ng/ml, cancer Gleason score is 6 or less (grade group 1), and the stage of cancer is as T1-T2a, N0 (no cancer can be seen in the lymph nodes) or NX (lymph nodes were not looked at, or the scans were unclear) and M0 (no metastasis), it is likely that cancer is completely localized within the prostate gland and has not spread to the lymph nodes or other parts of the body.

What are the treatment options for organ-confined prostate cancer and what are expected outcome?

Most of the organ-confined or localized prostate cancer grows very slowly, and therefore, might not need treatment at all. However, localized cancer needs to be monitored on a regular basis by active surveillance or watchful waiting.

Active surveillance is suitable for those patients with low risk prostate cancer. It can also be recommended for patients with intermediate risk prostate cancer, but not for high risk prostate cancer. During active surveillance patients should be monitored with regular blood PSA tests, MRI and biopsies. If any of these tests find changes from the previous tests then the patient might need treatment or complete removal of the cancer by surgery, or external beam radiotherapy or brachytherapy.

Watchful waiting is a way of monitoring a patient's prostate cancer that isn't causing any symptoms or problems. The objective is to monitor cancer growth and serum PSA levels to avoid cancer treatments. However, if symptoms develop, a hormone therapy to control the cancer growth could be used to help manage symptoms. Watchful waiting involves fewer tests than active surveillance and is generally suitable for patients with other health problems and not fit for surgery or radiotherapy. It might also be suitable for older patients whose prostate cancer isn't likely to cause any problem over their lifetime.

The main treatments for localized prostate cancer are: 1) surgery (radical prostatectomy), 2) external beam radiotherapy, and 3) brachytherapy. High-intensity focused ultrasound (HIFU) or cryotherapy can also be used but they are less common. Radical prostatectomy is a surgery to remove the prostate, including the cancer inside it. It can be done in three ways: laparoscopic (keyhole) surgery by hand or by robot-assisted and open surgery. External

beam radiotherapy (high-energy X-ray beams) can be used to destroy cancer cells from outside the body. In high risk patients, hormone therapy, also known as androgen suppression therapy is generally used to shrink the prostate and the cancer, making it easier to perform the surgery or to treat with external beam radiotherapy. There are many options such as, LHRH agonist or antagonist and fist and second-generation anti-androgens. Brachytherapy is a type of internal radiotherapy. It can be used together with external beam radiotherapy to give an extra dose of radiotherapy to the prostate. High-intensity focused ultrasound (HIFU) and cryotherapy use ultrasound to heat and destroy cancer cells. Cryotherapy uses extreme cold to destroy cancer cells. There's no overall best treatment for organ-confined prostate cancer. Each treatment has its own advantages and disadvantages and the type of side effects develop depends on the treatment being performed. Recently, a new technique known as CyberKnife or stereotactic body radiation therapy (SBRT), is used as a better non-surgical option for localized prostate cancer where it destroys tumors by providing beams of radiation at the cancer from multiple directions while sparing healthy tissues surrounding it. It is a very accurate, and effective treatment option for localized tumors in particularly hard to reach areas. Since it uses a combination of computers, image-guided cameras, and robotic technology, it can directly target tumor cells while sparing the nearby healthy tissue. Since there is no surgery involved, patients do not feel any discomfort during the treatment or experience minimal or no side effects. The patient needs less than five sessions, compared to traditional radiation therapy, which may require as many as 40 sessions. Moreover, it does not require anesthesia, incisions, or blood loss, therefore, recovery is quicker than traditional radiation therapy.

Conclusion

Because of the slow growth of prostate cancer, the majority of patients with organ-confined prostate cancer will survive. In fact, the majority of the patient may not need any treatments, watchful waiting or active surveillance will be good enough. However, some patients will need radical prostatectomy and targeted external beam radiotherapy (Cyberknife) is a very effective treatment options for organ-confined prostate cancer with a 5-year biochemical recurrence-free survival rate of about 90%. However, we must understand that prostate cancer is a multifocal disease. Currently

focal targeted therapy depends on the removal of prostatic foci based on saturation biopsy. So the potential risk of incomplete treatment might miss some cancer foci and might cause relapse of cancer again. Since we still do not have appropriate biomarkers for aggressive prostate cancer, it is hard to determine which organ-confined cancer foci will grow aggressively and which will be indolent. Therefore, future discoveries of better biomarkers for aggressive prostate cancer and technical advances in imaging tools would greatly improve the targeted therapy for men with organ-confined prostate cancer, so that organ-confined prostate cancer will no longer be a deadly disease.

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Chapter 59
How is castration-resistant prostate cancer now understood and treated?

Lara Rodriguez-Sanchez and Rafael Sanchez-Salas

According to the American Cancer Society, about one man in eight will be diagnosed with prostate cancer (PCa) during his lifetime. Conversely, "only" one man in 41 will die of the most common cancer among men, PCa. In other words, considering all patients diagnosed with PCa (most of them with local and potentially curable cancers), the probability of dying from this cancer 5 years after diagnosis is lower than 5%.

Indefinite hormonal suppression (through surgical castration by orchiectomy or chemical castration), whether continuous or intermittent, is the cornerstone of PCa treatment in two main scenarios: 1) when the disease is first diagnosed after it has already spread outside of the prostate to distant parts of the body or 2) when the disease progresses radiologically (appearance of metastasis) or biochemically (increase in PSA) after radical treatment with curative intent (Chapters 57, 58).

Up to 20% of PCa patients, metastatic or not, will progress to a more aggressive disease stage called castration-resistant PCa (CRPC). In contrast to the optimistic survival rates mentioned above, the median overall survival length of patients with CRPC is less than 5 years.

What does CRPC mean?

The National Cancer Institute describes CRPC as PCa that keeps growing even when the amount of testosterone in the body is reduced to very low levels. The name "CRPC" has been used synonymously with "androgen-independent PCa" ("AIPC") and "hormone-refractory PCa" ("HRPC"), but "CRPC" is the preferred and recommended term the PCa Working Group 2 (PCWG2) established.

How are androgens related to PCa?

The prostate stromal microenvironment (normal cells, molecules, and blood vessels that surround and feed a tumor cell), as well as epithelial prostate cells are both influenced by androgen activity. Therefore, the development and progression of PCa is intimately associated with what are considered "male hormones," specifically testosterone and its active metabolite, dihydrotestosterone (DHT).

The hypothalamic-pituitary–gonadal axis is the basis of normal androgen production in men. (Chapter 2). As Fig. 1 shows, the hypothalamus secretes cortical stimulation hormone (CRH) and luteinizing-hormone-releasing hormone (LHRH) (also known as gonadotropin-releasing hormone (GnRH)). These hormones induce the release of the adrenocorticotropic hormone (ACTH) and the follicle-stimulating hormone (FSH) along with luteinizing hormone (LH) from the anterior pituitary gland. ACTH will induce the production of dehydroepiandrosterone (DHEA), a precursor hormone for testosterone. LH stimulates the production of testosterone by Leydig cells in the testes, whereas FSH stimulates testicular growth and enhances production of androgen-binding protein by Sertoli cells. Circulating testosterone, either on its own or when locally converted to DHT by 5α-reductases, is able to bind to cytosolic AR, that translocates into the nucleus and acts as a transcription factor, binding to specific DNA sequences, leading to the expression and/or suppression of a variety of genes that can promote, among other events, tumor cell proliferation.

Resistance to castration appears after suppressive treatments via surgical castration, or chemical castration using 1) molecules that compete with androgens at the receptor level, antiandrogens (e.g., bicalutamide, nilutamide, flutamide, or cyproterone acetate); 2) LHRH antagonists (e.g., degarelix); or 3) LHRH agonists (e.g., leuprolide, goserelin, and triptorelin), which lead to the down-regulation of the LHRH receptors consequently suppressing LH and FSH secretion and hence testosterone production.

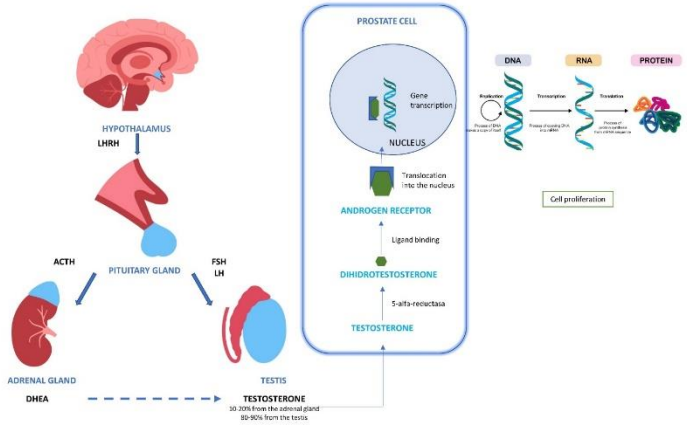

Figure 1. Hypothalamic-pituitary–gonadal axis and testosterone signaling pathway. LHRH; luteinizing-hormone-releasing hormone, ACTH; adreno-corticotropic hormone, FSH; follicle-stimulating hormone, LH; luteinizing hormone, DHEA; dehydroepiandrosterone.

How do PCa cells become androgen independent?

The way PCa becomes independent of androgen suppression is still a matter of debate, and some knowledge gaps remain; however, two main mechanisms have been described:

1. AR-dependent mechanism

Androgen signaling may persist despite androgen suppression due to mutation or amplification of AR genes and the consequent receptor overexpression, changes in expression of AR co-regulatory proteins, changes in expression of steroid-generating enzymes, or ligand-independent activation of AR via “outlaw” pathways.

2. AR-independent mechanism

Also known as “bypass” pathways. In this case, other non-androgenic ligands bind to their receptors, activating a signaling cascade independent of ARs leading to the transcription of genes involved in cell proliferation.

The understanding of the various ways tumor cells can continue growing regardless of the absence of androgens has been essential for the development of new therapeutic strategies to prolong the lives of patients who have reached one of the final stages of the disease.

What is the prognosis for patients with castration-resistant PCa?

Considering the natural history of PCa, this cancer begins as a localized disease. If the patient is diagnosed at this time, the therapeutic possibilities vary from active surveillance and focal treatment to radical treatment by either radical prostatectomy (with or without lymphadenectomy) or radiotherapy (with or without associated hormonal treatment). A percentage of these patients will experience a biochemical or radiological recurrence. As a first option, local salvage treatments with or without systemic treatment will be considered; however, such treatment will not always be possible, and an indefinite androgen blockage (intermittent or continuous) will be the basis of the treatment with the intention, at this point, not to cure the patient but to improve and prolong his life.

The median time from initiation of hormonal treatment to castration-resistant status is 2-3 years. The current specific criteria to define the status of castration resistance are: 1) serum testosterone levels less than 50 ng/dL or 0.7 nmol/L, and 2) 2 or 3 consecutive increases in PSA within two consecutive measurements separated by at least one week with a 2.0 ng/mL minimum increase over the starting value. In addition, documented disease progression based on findings from conventional imaging that includes bone scan and computed tomography scan using RECIST (Response Evaluation Criteria in Solid Tumors) are important diagnostic criteria.

CRPC can be categorized as either metastatic or nonmetastatic. Within approximately 1½ years, nonmetastatic CRPC will eventually spread. At this stage, currently the median overall survival expectancy is less than 3 years.

What are the treatment options for patients with CRPC?

We summarize therapeutic strategies for PCa in Fig. 2. In 1996, docetaxel was approved for the treatment of metastatic CRPC. Another taxane-based chemotherapeutic agent (drugs that interfere with microtubules blocking mitosis or cell division), cabazitaxel, was approved 14 years later for patients who have already received docetaxel treatment. In the same year, a type of therapeutic cancer vaccine, Sipuleucel-T, was launched as a new treatment option.

Due to advances in understanding the role of androgens in the various signaling pathways and cell proliferation, new therapeutic molecules have been developed in the last decade. These molecules are referred to as second-generation nonsteroidal AR inhibitors (ARis), androgen-receptor signaling inhibitors (ARSIs), and new AR-pathway-targeting agents (ARTA). We present the new AR antagonists and androgen synthesis inhibitors below:

Enzalutamide, apalutamide, and darolutamide

All three agents are novel anti-androgens with a greater affinity for the AR. These drugs have 3 complementary mechanisms of action:

1. competitive inhibition of androgen binding to the AR,
2. inhibition of nuclear translocation of the AR into the nucleus, and
3. inhibition of the AR binding to DNA, preventing the transcription of DNA into RNA.

Abiraterone

Its mechanism of action differs from those previously mentioned. Abiraterone blocks the enzyme CYP17, preventing the synthesis of testosterone in the testes, adrenal gland, and tumor cells.

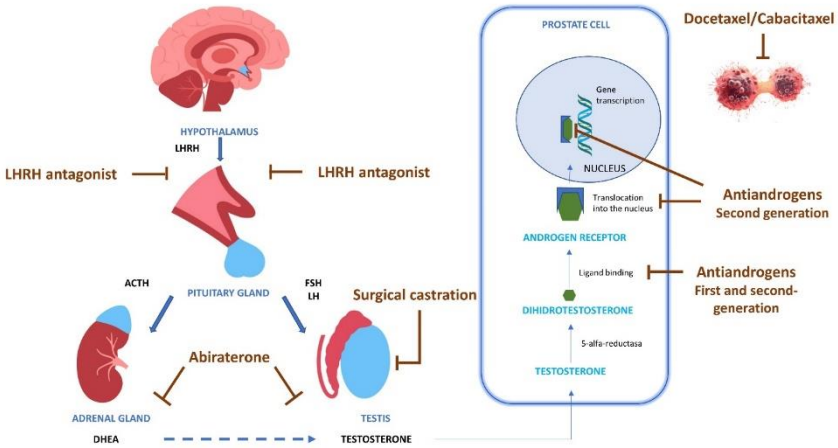

Figure 2. Therapeutic Strategies in PCa
LHRH; luteinizing-hormone-releasing hormone, ACTH; adrenocorticotropic hormone, FSH; follicle-stimulating hormone, LH; luteinizing hormone, DHEA; dehydroepiandrosterone.

Radium 223

A radiopharmaceutical that selectively targets bone metastases with alpha particles. This treatment is suitable for patients with symptomatic bone metastases but without known visceral disease or lymphadenopathy >3cm. It cannot be used in combination with chemotherapy or ARTA.

Poly(ADP-ribose) polymerase (PARP) inhibitors

PARP enzymes aim to identify and repair DNA damage. Therefore, PARP inhibitors will have a cytotoxic effect.

- Olaparib is indicated in the case of a deleterious germline or somatic homologous recombination repair (HRR) gene mutations.
- Rucaparib must be considered in the case of BRCA1 or BRCA2 mutations.

Pembrolizumab
This is a type of immunotherapy that blocks the programmed-cell-death protein (PD-1) located on the surface of T-cells. PD-1 induction is a kind of adaptive immune resistance because this protein's bond with its corresponding ligand (PD- L1) helps keep T-cells from killing other cells, including cancer cells. This compound has been approved for patients with microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) solid tumors.

Lutetium-177 (177Lu) prostate-specific membrane antigen (PSMA)-617
A radioligand therapy that delivers beta-particle radiation to PSMA-expressing cells (characteristics of PCa), inducing their death. In 2022, the US FDA approved the use of this drug for the treatment of patients with prostate-specific membrane antigen (PSMA)-positive mCRPC who have been treated with androgen receptor pathway inhibition and taxane-based chemotherapy

Sipuleucel-T
An immunological agent, may be administrated in patients with asymptomatic or minimally symptomatic nonvisceral metastases, a good performance status and a life expectancy superior to 6 months.

What are the treatment options based on the stage of the disease?

It is important to bear in mind that first-line androgen-deprivation therapy must be continued to maintain castrate serum levels of testosterone <50 ng/dl. The treatment chosen depends on the presence of metastasis, the PSA doubling time (the shorter the time, the greater the risk of metastasis), and the previous received treatment.

For non-metastatic Castration-Resistant PCa
if PSA doubling time >10 months, then continuous monitoring is the preferred option. If PSA doubling time <10 months, then use apalutamide, darolutamide, or enzalutamide

For non-metastatic Castration-Resistant PCa, four therapeutic approaches are possible

1. If no prior docetaxel and no prior novel hormone therapy was

used, then any of these are options: Abiraterone, Enzalutamide, Docetaxel, Radium 223, Sipuleucel-T;
2. If no prior docetaxel was administered, but prior novel hormone therapy was used, then any of these may be used: Docetaxel, Sipuleucel-T, Cabacitaxel/Carboplatin, Olaparib, Rucaparib, Pembrolizumab, Radium-223;
3. If prior docetaxel was used, but there was no prior novel hormone therapy then any of these may be used: Abiraterone, Enzalutamide, Cabazitaxel, Cabacitaxel/Carboplatin, Pembrolizumab, Radium 223, Mitoxantrone (palliative in symptomatic patients who do not tolerate other therapies);
4. If Prior docetaxel and prior novel hormone therapy were used then few options are available because chemotherapy and new AR-pathway-targeting agents, the mainstays of treatment for CRPC, have already been used. Nevertheless, in certain circumstances, patients may receive any treatment that has not been employed (e.g., Cabacitaxel/Carboplatin, pembrolizumab, radium 223, PARP inhibitors). In this scenario, Lutetium Lu-117-PSMA-617 may be considered under certain circumstances.

Although therapeutic approaches for the treatment of CRPC were stagnant for many years, we have witnessed a burst of novel approaches that have proven effective in prolonging the life of CRPC patients. Yet, none of these valuable approaches are providing a cure for this cancer. Refinement of these therapeutic approaches may further increase life expectancy, but we await the development of new, individual patient targeted approaches to provide avenues for curing CRPC.

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Chapter 60
What is an andrologist now? What does an andrologist do? What will an andrologist do in the future?

Peter N. Schlegel

An andrologist is a physician or scientist involved in the study of diseases affecting the male reproductive tract, including the prostate, an individual who seeks to understand how the male reproductive system works, or an individual who provides clinical evaluation and/or intervention for male fertility or sexual function. An andrologist can have a variety of different backgrounds, ranging from standard college training in a scientific discipline that is supplemented by laboratory training to college, doctoral, and postdoctoral education for researchers or advanced laboratory directors, or clinical training including medical school as well as residency and fellowship training for physician who work as clinical andrologists.

The laboratory-based andrologist often works with clinical samples, performing semen analyses including sperm concentration, motility, morphology and parallel studies on the semen sample. They may also process or prepare semen samples using simple or advanced sperm washing and selection techniques that help to identify the best sperm for intrauterine insemination, IVF or ICSI. Additional tests on sperm may look at characteristics of sperm DNA including DNA damage or chromosomal assessments as well as functional assessments of sperm.

Andrologists may also qualify as laboratory directors if they have doctoral level training. Laboratory directors are responsible for overall andrology laboratory operations, manage laboratory staff and have a critical role in establishing and maintaining quality control, human resources and even development and/or validation of new tests for sperm or semen assessment. They often perform the procedure of intracytoplasmic sperm injection, including preparation and assessment of eggs as part of that procedure if the andrology laboratory is linked to an IVF (in vitro fertilization) or embryology laboratory. Andrologists in this setting are often trained as embryologists as well.

Investigative andrologists are researchers who study the underlying processes of regulation of the hypothalamic-pituitary-testicular axis, sexual development, puberty, reproductive aging, steroidogenesis, spermatogenesis, and the effects of drugs and environmental conditions/toxicants on every aspect of the male reproductive system. These andrologists typically work in a university or non-profit setting oriented toward research.

Andrologists play an important role in veterinary schools and large animal facilities such as zoos where they seek to understand the wide range of male reproduction processes and practices within the animal kingdom, preserve male germ cells and treat pathological conditions in animals, whether as pets, on farms or in zoos.

Clinical andrologists may be physicians with a background in internal medicine/endocrinology, urology, or related fields. As such, they perform evaluation and management of male infertility patients. This may include microsurgery or other surgical interventions for patients. Residency training may require 3-8 years, including fellowships after medical school to reach this level of subspecialty expertise. Since andrology also includes study and treatment of prostate problems and/or sexual medicine, some urologists function as andrologists. Clinical andrologists also can be actively involved in sexual medicine practice.

The future of andrology is strong and broad. We increasingly understand how genetic variants derived from the male may affect reproduction, including fertilization, embryo development and the health of a child. The condition of male infertility has also been recognized as a marker for the overall health of a man, and the mechanisms by which impaired sperm production affects a man's future health are increasingly being elucidated (Chapter 64).

Sperm are recognized as providing the microtubules that direct embryo development, and sperm-related factors such as sperm DNA fragmentation can affect maintenance of a fetus even after embryo implantation. Sperm factors affecting the health of the offspring include paternally-derived genetic and/or epigenetic variations that increase the risk of conditions such as autism, schizophrenia and other developmental disorders as well as neoplastic risk.

Each of these andrological conditions and their contributions to reproduction will expand as further insight into the mechanisms by which sperm and male factors affect subsequent reproductive action are elucidated. The mounting research efforts to develop safe and effective male contraceptive will allow the male partner to play an active role in the contraceptive practice in couples. New insights will

allow for further interventions to ameliorate such male factors. The ongoing capability of scientists to make observations of sperm contributions during assisted reproductive treatments further increase the recognition of male factors in reproduction. The ability of regenerative technologies to enhance development of the male gamete outside of the testis will both enhance our understanding of spermatogenesis and the ability to treat infertile men with no current options for therapeutic intervention. The future of andrology is indeed rich with opportunities for scientific advances.

Chapter 61
What is the role of the andrologist with respect to the LGBTQQIA2S+ community?

Alan W. Shindel

The term andrologist is derived from the Greek words "Andros" (the genitive of aner, meaning "man") and "logy" (a derivative of the term meaning "word", typically attached to root words to convey the study of a given subject). Andrology is hence the "study of men" and in common medical parlance hence refers to evaluation and management of issues affecting men's health, most commonly disruption of reproductive and sexual function.

Andrology has focused on people who have the biological features commonly associated with masculinity (e.g., Y chromosome, prostate gland, penis, testes) as well as secondary sex characteristics related to effects of androgens (e.g., hirsutism, increased muscle mass, enlarged larynx). While these structures may be conceived of as andrological and are typically considered indicative of male sex, individuals who have them may not necessarily conceive of themselves as male or masculine. Other individuals may identify as masculine or having masculine traits but have been born with a chromosomal complement of 46XX and/or ovaries, a uterus, and a vagina.

These examples illustrate the important distinction between sex (i.e., the physical structures and organs one is born with) and gender (i.e., the concept of self as a being with traits and characteristics associated with a particular gender identity). These concepts are also distinct from sexual orientation (i.e., what physical or behavioral features an individual is attracted to in sexual or romantic partners). Stated another way in colloquial English, gender identity is "how you identify yourself", sexual orientation is "with whom do you have sexual or romantic relationships", and sex is "genital structures and chromosomal content that can (typically) be determined at birth."

The past several decades have seen an explosion of advocacy and increasing visibility for people who endorse sexual interest in same-gender partners (i.e., lesbian women, gay men, and bisexual

persons of any gender, oftentimes grouped together as a group referred to as "LGB"). Issues of gender identity have been more prominently recognized over the past decade. A growing number of individuals endorse gender identities that are not congruent with the traditional gender identity associated with their natal anatomy. Some persons may identify with the "opposite" gender, i.e., a person with natal male sexual organs identifying as female or vice versa. Such persons may identify as transgender, a more appropriate term than the outmoded and pejorative term transsexual. A "T" to represent "transgender" was added to the term "LGB" to create the familiar "LGBT" acronym.

A growing number of persons reject the entire concept of a gender binary and endorse identities outside of the classic male-female dichotomy. Examples include "genderfluid", "gender non-binary", "gender-queer", "agender", "two spirit", or any one of a number of other monikers outside the classic dyad of male and female. The traditional continuum of sexual attraction promoted by the sex researcher Alfred Kinsey, ranging from strictly heterosexual to strictly homosexual with variable degrees of mixed attraction between these extremes, has also been challenged. A small but increasingly visible group of individuals exist who report no or low sexual attraction to partners of any gender. These persons may be further subdivided into "asexuals" ("aces", i.e., not interested in sexual relations but interested in romantic companionship) and "aromantics" ("aros", i.e., not interested in sexual or romantic relationships).

It is increasingly clear that the concerns and needs of these various groups of diverse sexual orientation and gender identity cannot accurately be described as simply "LGBT". As such, the acronym has now been expanded to include Questioning, Queer, Intersex, Asexual, and Two Spirit persons (LGBTQQIA2+). This expanding acronym is more precise in the sense that it better characterizes constituent groups. However, expansion of the group risks conflating unique groups of persons that may not have much in common aside from identifying with a gender different from what their natal sex would predict, having a non-heterosexual orientation, and/or having experienced societal recrimination for these reasons.

Concepts of sexual orientation and gender identity are controversial and emotionally charged. Increasing prominence of LGBTQQIA2+ persons in public life has led to a legal and political backlash from persons and groups with more conservative and traditional views on sexuality and gender. As researchers and

clinicians interested in reproductive and hormonal biology we are beholden to concepts of fact and beneficence in the work we do on behalf of our patients and society as a whole. We must always strive to put scientific integrity and the well-being of our patients (for those of us who are clinicians) at the forefront of what we do. This is not to say that an orthodoxy of thought regarding sexual orientation, gender, and their expression is required of andrologists, nor that any side in the fierce and emotional debates about these topics has a monopoly on the more nebulous and philosophical concept of truth.

Persons who identify with one or more of the constituent groups of the LGBTQQIA2+ community have often experienced discrimination, sometimes from healthcare providers. These groups often also have been poorly represented in clinical research and as researchers beyond risk management strategies for prevention of sexually transmitted infections including HIV. What, then, are our obligations as clinical and research andrologists to the various persons belonging to LGBTQQIA2+ communities?

Gay and Bisexual Cis-gender Men

The role of andrology researchers and clinicians is most easily understood in terms of how we may serve the needs of gay and bisexual men (GBM) who are cis-gender. These groups identify as men and have male sexual/reproductive organs. As basic biological processes do not differ between heterosexual and non-heterosexual men, andrology practitioners can meet the needs of GBM by addressing common andrological concerns such as hypogonadism, isolated low serum testosterone, prostate cancer, erectile dysfunction, decreased libido, Peyronie's Disease, and other issues common to people of male sex. While the andrological problems may not differ, cultural and behavioral practices in GBM may portend health risk factors that differ from heterosexual cisgender men. For instance, HIV infection is more common in GBM, and HIV infection is in turn associated with higher risk of the andrological syndrome of hypogonadism.

From the clinical perspective, it is of primary importance not to assume heterosexuality in men presenting for healthcare. Tactful and sensitive inquiry into sexual orientation and sexual/reproductive health concerns is a requisite for optimal clinical care. Clinicians should avoid making assumptions about the gender of a patient's sexual partner(s) or their marital status. Patients who are able to disclose their sexual orientation and practices to their

healthcare provider are more likely to receive appropriate care so this element of clinical disclosure is essential.

Clinical interventions that work for heterosexual men will generally be efficacious for their GBM peers and should be offered as clinically appropriate. The risks of therapies are also likely to be similar; however, the impact of these adverse outcomes may differ. Sexual practices are variable in GBM so clinicians should make efforts to explain how specific therapies may have greater impact in this population. For instance, although receptive anal sex is not ubiquitous in GBM but does occur more frequently than in heterosexual populations. For this reason, the effects of surgical or radiation-based treatment on prostate and/or rectal sensation should be considered when counseling GBM with prostate cancer who engage in receptive anal sex.

Liberalizing of laws and social norms has seen a marked growth in the number of GBM pursuing paternity, including fathering biological children. Clinicians caring for GBM should inquire about any desire or plans for biological paternity. Biological paternity requires fertilization of an ovum with the man's sperm and support of the developing fetus in a gestational carrier (e.g., a paid surrogate, family member, or other individual with a uterus who may also be interested in a future parenting role). Flexibility and a desire to accommodate what may be a non-traditional conception, pregnancy, and future family structure is essential to the clinician providing care for these men.

From the research perspective, explicit inclusion of GBM in research should be a priority. While we do not have evidence that basic biological processes substantially differ in GBM versus their heterosexual peers, the sociocultural milieu of GBM may alter the efficacy of therapy. A more complex and potentially dangerous line of research would be to understand the biological underpinnings of same sex attraction. While such information might be of genuine scientific interest, legitimate concerns exist about how such knowledge may be abused to try and "cure" GBM of same sex attraction or legitimize prejudice.

Transgender Women and other non-male Assigned Male at Birth Persons

To reiterate, a transgender woman is an individual with natal male anatomy (i.e., born with a penis and testes) who identifies as a

woman. Some persons with natal male anatomy may not identify with either male or female.

Transgender women are at risk for common "male" conditions such as testicular cancer, benign prostate hyperplasia, and prostate cancer. Appropriate screening and treatment of these conditions should be offered. Transgender women may feel distress at having problems with "male" organs and as such may not feel comfortable seeking out appropriate screening and testing. It is essential that andrologists show support. An important element of this can be utilizing the patient's preferred terms for parts of their anatomy; this is often (but not always) the anatomical word such as "phallus" or "penis" but may be an alternative such as "clitoris" or a non-anatomical term.

Some transgender women may desire fertility preservation. Depending on their puberty status, it may be possible to bank semen for future use in assisted reproduction. Transgender women on gender affirming therapy often have suppression of sperm parameters so, when possible, banking should be initiated prior to gender affirming hormone therapy. If sperm cannot be procured from ejaculated semen, sperm aspiration or a harvesting procedure may be utilized.

Sexual expression in transgender women may or may not involve use of their penis; transgender women who engage in insertive sex or other forms of penile stimulation may develop issues of erectile dysfunction (ED) that impair their capacity for sexual satisfaction. Standard therapies for ED (e.g., phosphodiesterase type 5 inhibitors, intracavernous injections of vasodilator medications) may be offered to these patients.

Transgender women may have gone through puberty and experienced development of male secondary sex characteristics. These traits may be very distressing to transgender women; this has led to a push from many corners to promote the use of puberty blocking drugs for pre- or early-pubescent children who identify as transgender. These treatments may be of psychological benefit in many cases, but the data on safety and long-term repercussions remain incomplete. Further research will help better define the optimal criteria and type of puberty blockade. The expertise of andrologists in understanding issues of testosterone biology may be of great service in these research efforts to optimize the care of transgender persons.

Natal female individuals

The role of the andrologist in the care of the transgender man (i.e., natal female but identifying as male) is at this time poorly defined. Andrologists are clearly in the best position to understand the role of androgens in the human body. From a research perspective, andrologists should study the long-term effects of high dose testosterone supplementation in the natal female body. Clinical andrologists are well-qualified to offer transgender men gender-affirming hormone replacement therapy with testosterone supplementation by any one of a number of evidence-based modalities.

The role of the andrologist is not well defined in the care of cis-gender women, regardless of sexual orientation. There may be some interactions if a cis-gender woman is involved with a GBM planning paternity as a gestational carrier

Conclusions

Androlologists are by definition knowledgeable and interested in issues of sexuality and hormones. This interest is well suited to the research and care of individuals who exist outside the classic binary of “male” and “female”. Our knowledge and skills can also be used to serve other groups (e.g., GBM) who have been historically marginalized by the medical and research establishments due to their sexual orientation.

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Chapter 62
How is social media changing andrology?

Heather E. Fice

If you open the popular video social media app, TikTok, #Andrology has millions of views. Some of these videos are from physicians, speaking about men's health issues in an educational manner, some of these videos have keen graduate students speaking about their research, and some of these videos offer reproduction facts that are completely unfounded in health research. This presents us with a novel challenge in providing andrology care: the influence of social media.

In 2022, it is typical of individuals with a health concern to do a quick online search for their symptoms prior to consulting a physician. This can mean that patients are well informed upon consultation, or that their healthcare journey begins and ends with an online search. As men have a baseline reluctance to access healthcare, they would often rather turn to various online sources including online resources, social media or the 'participatory web'. Online resources include websites that offer health information in an accessible format, such as WebMD, governmental websites, and other health oriented organizations. This information is most frequently accessed by individuals over the age of 55. This information is posted with intent, and curated by practitioners and health experts. However, the participatory web is a collection of websites on which users are able to share their health experiences with little to no moderation from licensed healthcare providers. Reddit, TikTok, Twitter, Instagram, Facebook, and other small online forums are websites or apps that make up the abundance of easily accessible information and personal testimonies. People are able to make their own posts, comment, share other posts, and otherwise engage with content of interest. You can follow pages that suit you, and join communities that fit your needs or desires. Social media websites and apps are most commonly accessed by individuals under 40 years of age.

Within the field of andrology, the web plays an especially prevalent role as many health concerns are stigmatized or taboo,

such as: erectile dysfunction, sexually transmitted infections, human immunodeficiency virus (HIV), infertility, low testosterone or Peyronie's disease (and more). Men are ashamed or face barriers in discussing these issues with their healthcare providers or advocating for their care. These sociocultural barriers are made worse by the overall access to care, including physical proximity and insurance coverage, or cultural acceptance of specific disorders.

Though using the internet for health concerns may sound alarming, there are both pro's and con's to having easily accessible banks of information about health issues on social media sites, popular mobile technologies, and other participatory websites.

What are the benefits?

Having factual information about men's health on social media has many potential benefits. Primarily, it will have major benefits regarding patient and public education. Individuals are now able to read, often in laymen's terms about men's health and andrology. The stigma associated with disorders can be dispelled through online campaigns and educational content accounts. There have been great efforts made on Instagram in reducing stigma of herpes simplex virus carriers, for example. Patients on the participatory web have access to in depth explanations of potential disorders, symptoms, treatments and diseases.

Individuals at risk of certain disorders may benefit from outreach programs through social media, with awareness being brought to issues through trending hashtags or accounts. For sexually transmitted infection centric education, social media efforts have been game-changing. The Get Yourself Tested campaign was aimed at all sexually transmitted infections, and gained large support on social media. The attention led to increased testing in nearby centres. There have also been campaigns used specifically to promote HIV testing of men who are statistically more at risk of contracting HIV, including men who have sex with men, and black men. Patients can be made aware, become more informed, and access care armed with as much knowledge as possible. There is an element of empowerment in knowing which questions to ask physicians, and understanding potentially complex medical jargons.

In addition to finding information people are able to find and form communities with one another to build solidarity in certain diagnoses or symptoms. This opens global avenues for peer support that can remain virtual, and anonymous if desired. In the case of

fertility status or infertility diagnosis, peer-support is invaluable for men experiencing infertility. Men reportedly face distress due to diagnosis as they feel somehow 'less', and they feel unable to share their emotions with their partner as they must also provide support. The value in peer-support is due to the unique psychological challenges that men face, and the fact that many men do not seek mental health care as a result of diagnosis. In a peer-support context, men are able to validate and normalize their experiences with fertility status. The desire for online, anonymous support has been reportedly high for men, with greater desire from men who are persons of colour or lower income.

What are the risks?

The major drawbacks associated with social media coming into the field of andrology are the quality of information, and the lack of generalizability in healthcare. It can be challenging to provide information that covers all potential cases, in all potential populations. Clinicians know that medical care is not often a one size fits all field, and what works for one person may be entirely different from what is needed by another. When patients hop online to find answers to their questions, search their symptoms, and examine treatment options, they may be faced with stories from other patients who have different needs. Though that community provides valuable support, there exists a line where the information shared is no longer helpful. Personal testimony holds the potential to unfortunately bias perceptions when accessing information, as often people who share their own journeys have had overwhelmingly positive or negative experiences. With larger social media sources, such as TikTok, Facebook, Instagram or Twitter, there is a large bias towards successful medical interventions. On this note, men who have undergone fertility care are often quick to share stories of success upon the conception of a child. These participatory websites are often not monitored by healthcare practitioners or researchers. Moderators are particularly valuable in cases where individuals may over generalize their disorders or diseases. Moderation is also useful, as dangers obviously arise when that information shared online is invalid. This presents an issue when individuals are not critical of what they read, or they are not addressing their concerns directly with a practitioner. Fake health news spreads very quickly on websites like Facebook, which may also cause alarm in populations who need not be concerned. It spread like wildfire on

social media in 2020 that the vaccines against SARS-CoV-2 would make men infertile, though not founded in scientific or clinical research. Zailia et al., (2020) reported that as much as 44% of the information on infertility was not grounded in research and was misleading and inaccurate. Scarily, there was no difference in the engagement based on the quality of the information.

A final caution is when social media information is being used in place of health care. When individuals are empowered to take their care into their own hands, this can lead to dire consequences. A study was done on the reasoning behind self-medication with testosterone, or image and performance enhancing substances, in men on social media forums. The men being studied reported that they were able to self-diagnose with low testosterone, and subsequently able to dose themselves with anabolic steroids based on dosing regimens that had worked for others. One man went so far as to call it 'broscience'. This highlights many of the dangers associated with social media in andrology, as we cannot begin to unpack the potential concerns with regard to self-treating with black-market testosterone.

Conclusion

The power of social media in andrology is immense, with both great and awful sides to this coin. As clinicians, researchers, and those best educated in andrology, it may become our duty to be proactive as moderators in this space. We may be tasked with bringing facts to social media, dispelling myths, and doing online education using these platforms to prevent the dangers that may arise and use the reach of social media to our advantage.

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Chapter 63
What is the impact of COVID-19 and vaccines for COVID-19 on sexual and reproductive health?

R. John Aitken

In 2019 the world was gripped by a COVID pandemic that has, to date, infected 500 million individuals and taken over six million lives. This virus is generally thought to target the lungs, kidney and heart, although there is growing evidence that it can also invade the reproductive system, particularly in the male. As a result, there is increasing concern over whether this virus can have a detrimental impact on the male reproductive function, whether such an attack can seriously impact semen quality and/or the biogenesis of androgens, whether such impacts induce transient infertility or sterility, and whether vaccination might be a help or a hindrance in managing the reproductive impacts of this disease. This article summarises the data currently available on this topic and provides a snapshot of our current understanding of how COVID-19 infection influences male fertility.

COVID-19 infection of the male reproductive tract

Initial awareness of the male reproductive system's vulnerability to COVID-19 attack was predicated upon proteomic analyses indicating that the male germ line contains the entire repertoire of proteins needed to facilitate the binding and incorporation of viral particles. Thus, the viral spike glycoprotein (S) that gives this corona virus its name, has a particular affinity for ACE2 (angiotensin-converting enzyme 2), which is highly expressed on the surface of several cell types in the testes including Leydig cells, Sertoli cells, macrophages and the germ line. The presence of ACE2 on the surface of human spermatozoa is thought to be responsible for the generation of angiotensin 1-7 at the cell surface (Fig. 1). This endocrine product of ACE2 processing, is potentially important to spermatozoa because it binds to the MAS receptor, thereby activating phosphoinositide 3-

kinase (PI3K) which in turn phosphorylates a number of proteins that are essential for sperm survival, preventing these cells from defaulting to an apoptotic fate (Fig. 1). A COVID-19 attack on ACE2 is therefore predicted to result in proteolytic cleavage of this enzyme, leading to a loss of PI3 kinase activity and a consequential impairment of sperm viability and function (Fig. 1). Furthermore, since both Leydig- and Sertoli- cells are also known to express the ACE2/angiotensin 1-7/MAS receptor/ PI3 kinase pathway, any impact of COVID-19 infection on these cells could involve the induction of apoptosis/loss of function via similar mechanisms.

Actual fusion between the virus and a target cell requires the presence of the protease, TMPRSS2, to cleave the viral spike protein (S) at the S1/S2 boundary or within S2 subunit, thereby removing the structural constraint of S1 on S2, and releasing the internal membrane fusion peptide (Fig. 1). TMPRSS2 protein is abundantly expressed in the prostate, but can also be found in the epididymis, Leydig cells, spermatids and spermatogonia. This protease is also known to be present in seminal prostasomes that transfer their contents, including proteins, to the spermatozoa following ejaculation. Via this mechanism the incorporation of TMPRSS2 into the sperm plasma membrane has been proposed. Furthermore, a close examination of the sperm proteome reveals the presence of related proteases, TMPRSS11B and TMPRSS12 as well as FURIN in these cells, all of which have the potential to facilitate viral entry. The presence of these activating proteases as well as ACE2 in the sperm plasma membrane would be expected to allow the COVID-19 virus to bind to the cell surface and ultimately fuse, either in the testes or during the prolonged sojourn of these cells in the epididymis. On this basis it has been argued that spermatozoa would well act as a vector for COVID-19 infection making this, potentially, a sexually transmitted disease. Notwithstanding such theoretical considerations, at present, there is no convincing evidence to demonstrate that this is the case. COVID-19 infection seems to be spread primarily via an airborne means of dissemination. However, given the publication of occasional reports describing the presence of viral RNA in human semen, we cannot completely exclude the possibility that when viral loads are extremely high, then sexual transmission of COVID-19 might occur.

Whether or not the virus can infiltrate human semen and remain active, the testes can certainly become heavily infected with this virus and is even thought to be a sanctuary for COVID-19 replication.

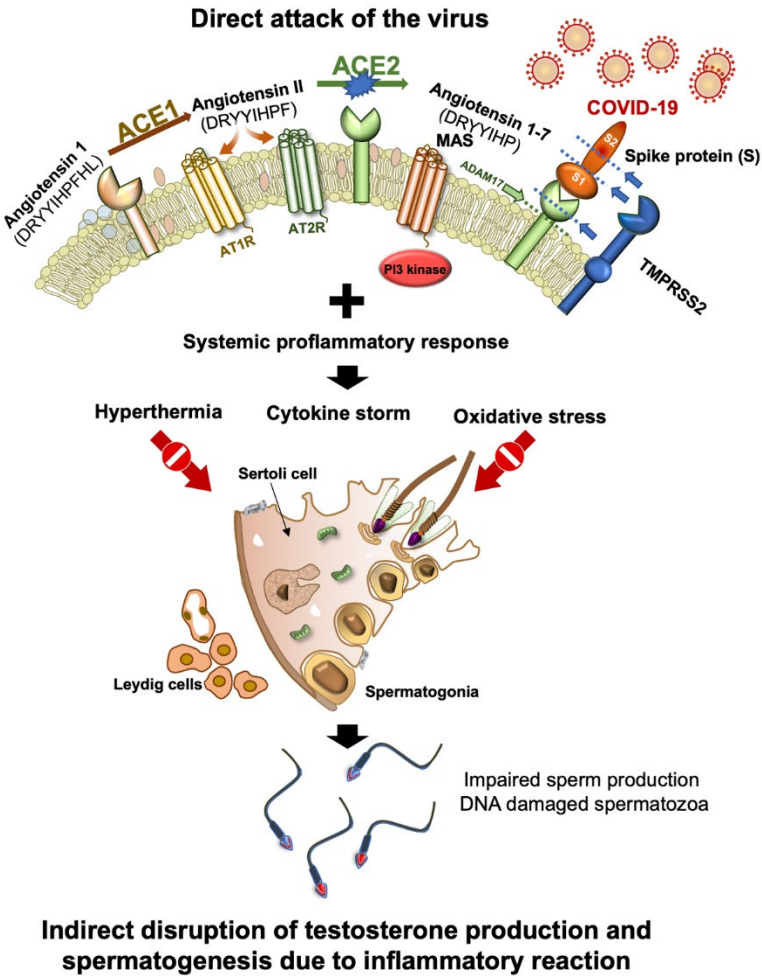

Figure 1. Mechanisms by which COVID-19 can impact male fertility. Several key cell types within the reproductive system (Sertoli cells, germ cells, Leydig cells, epididymal epithelia, prostate gland epithelia) are vulnerable to viral attack because they express ACE2, which is targeted by the viral spike glycoprotein (S). Disruption of ACE2 will lead to a local loss of angiotensin 1-7, which is a key ligand for the MAS receptor which drives PI3 kinase activity. In the case of spermatozoa, a loss of PI3 kinase activity will cause these cells to default to the intrinsic apoptotic pathway. The presence of the viral fusion transmembrane protease, serine 2 (TMPRSS2) protease on the surface of

reproductive cells allows COVID-19 to enter the cell and initiate viral replication. Within the testes, both spermatogonia and macrophages appear to be major viral factories supporting high levels of replication and acting as a reservoir ensuring the prolonged supply of active virions. In addition to the direct impact of viral infection on reproductive function, COVID-19 also induces a pro-inflammatory state characterized by a cytokine storm accompanied by high levels of oxidative stress and hyperthermia. These conditions actively suppress spermatogenesis and disrupt testosterone production by the Leydig cells. This combination of direct and indirect actions on the part of COVID-19 has a devastating effect on male reproductive function, which generally abate once the infection resolves.

Thus, there is recent evidence to indicate that the virus gains access to both testicular macrophages and spermatogenic cells and can actively replicate at these sites forming an intratesticular repository of active virus, which may prolong the impact of COVID-19 infection on male reproductive function. Furthermore, the inward migration of infected macrophages from distant sites such as the lung may represent one of the key mechanisms by which COVID-19 infection is delivered to the testes - acting as Trojan horses and exploiting the capacity of this organ for immune tolerance.

The pro-inflammatory state and male reproduction

In addition to the direct effects of the virus on the male reproductive tract, it is also probable that male fertility is impacted by the cytokine storm that accompanies infection by COVID-19. The latter triggers a hyperactive immune response associated with the release of key cytokines (for example, IL-1β, IL-6, IL-8, IL-10, TGF-β, TNF-α, IFN-αand IFN-γ) that, in turn, generate a proinflammatory state. Several of these cytokines (for example IL-10, IL-6, IFN-γ, and TNF-α) are known to signal the development of inflammatory conditions that are harmful to semen quality and male fertility. Furthermore, certain cytokines, such as TNF-α, can have a direct impact on the functionality of human spermatozoa. In addition, the induction of an inflammatory state is associated with an increase in testicular temperature and the associated induction of oxidative stress. The deleterious impact of such stress on testicular function reflects the vulnerability of Leydig- and germ- cells to both lipid peroxidation and the induction of oxidative DNA damage. Furthermore, oxidative stress is known to have a negative influence on the integrity of the

blood testes barrier, which will only serve to reinforce COVID-19's disruptive effect on spermatogenesis.

Observed clinical impacts on male reproduction

When infection does occur, it seems to result in the rapid impairment of sperm quality decreasing sperm number and motility in the ejaculate and increasing the incidence of morphological abnormalities in these cells. In addition, COVID-19 infection has been found to induce profound changes in overall testicular architecture and function including thickening of the tunica propria, germ cell apoptosis, Sertoli cell barrier loss, haemorrhage, angiogenesis, Leydig cell inhibition, inflammation, and fibrosis. Even a relatively mild infection that does not require hospitalization, has been linked with the rapid induction of azoospermia (complete absence of spermatozoa in the ejaculate) in longitudinal studies on single individuals monitored before, during and after COVID-19 infection. In addition, COVID-19 infection may result in reduced testosterone levels in infected males, accompanied by increases in pituitary gonadotrophin (LH and FSH) output. These observations are consistent with the direct suppression of Leydig cell function rather than an indirect action mediated by changes to the hypothalamic-pituitary axis. Happily, such impacts on sperm production and function, as well as testosterone biosynthesis, seem to be readily reversible, with several studies documenting a return to normality within a matter of weeks. Notwithstanding such observations, there are also clear inter-individual differences in the rate at which normal reproductive function returns, that will have to be carefully monitored in future studies. Furthermore, long term impacts on male fertility associated with sperm DNA damage and/or the generation of anti-sperm antibodies cannot be excluded at this stage.

Relative importance of viral infection and oxidative stress

Present data suggest that the negative impact of COVID-19 infection on male reproductive function involves a combination of direct cellular damage induced by the virus and indirect damage mediated by the accompanying inflammatory reaction and oxidative stress. The apparent capacity of the virus to rapidly induce complete azoospermia may reflect its ability to replicate within spermatogonia, leading to high

levels of germ cell apoptosis and the cessation of sperm production. The suppression of spermatogenesis may also involve a direct viral attack on Leydig- and/or Sertoli- cells, both of which have been found to harbour viral particles following infection.

Indirect mechanisms are also important in determining the reproductive response to COVID-19 infection. It is well known that a range of viral infections that do not directly target the germ line (e.g. mumps or HIV) can have a disruptive impact on male reproduction as a consequence of inflammation and the resulting induction of oxidative stress and hyperthermia in the testes. The oxidative stress associated with COVID-19-induced inflammation induces significant oxidative DNA damage and lipid peroxidation in spermatozoa, that appears to persist, even when normal semen parameters have been restored. The oxidative stress associated with COVID-19 infection has also been found to reduce testicular glutathione levels and precipitate a concomitant increase in ROS generation and apoptosis in the male germ line. Interestingly, treatment with antioxidants such as N-acetyl cysteine has been shown to significantly improve semen quality in COVID-19 patients. In light of these data, a systematic, case-controlled analysis of the protective impact of antioxidant therapy during and after COVID-19 infection is certainly warranted.

Impact of anti-COVID vaccines

Several studies have examined the impact of anti- COVID-19 vaccination on semen quality and all are unanimous in finding no discernible impact on spermatogenesis or testosterone production. In addition, the fertilizing potential of spermatozoa following vaccination was found to be unimpaired.

Conclusions

The COVID-19 virus impacts male fertility via two major mechanisms : (i) direct interaction of the virus with ACE2 on the surface of key reproductive cell types including Sertoli cells, Leydig cells and the germ line, and (ii) the generation of a cytokine storm and the subsequent appearance of a proinflammatory state associated with raised testicular temperature and clear evidence of oxidative stress. In a vast majority of patients, the impact of the virus on semen quality and testosterone output is sudden, and occasionally severe, but generally reversible within a few weeks.

However, the persistence of oxidative DNA damage in the germ line and the occasional presence of anti-sperm antibodies may have long term implications for the fertility of a small minority of patients. There is limited evidence to suggest that antioxidant therapy might be beneficial in counteracting the impact of the virus on male fertility although additional studies will be required to confirm the therapeutic benefit provided by such therapy and to refine the precise nature of the antioxidants to be used in this context. There is also consistent evidence to indicate that COVID-19 vaccines have no impact on male fertility per se. Indeed, by controlling the onset of severe febrile disease, vaccination should do nothing but support male reproductive health in the face of this pernicious virus.

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Chapter 64
Is male reproductive dysfunction a window on general male health?

Federico Belladelli and Michael L. Eisenberg

Approximately 15% of all couples are infertile with a male factor in around 50% of cases. Importantly, there is a growing body of evidence suggesting that male factor infertility (MFI) (conventionally defined as men with impaired spermatogenesis, Chapter 32) may be a potential biomarker for overall health, as it has been associated with the development of cancer. In addition, disorders such as diabetes, metabolic syndrome, and obesity have been detected at a higher rate among patients with MFI. Furthermore, other studies provide data on a link between infertility and cardiovascular disease (CVD). Moreover, large population studies suggest that there is a higher mortality risk for patients diagnosed with MFI. While the etiology of the association between health and fertility is uncertain, investigators have hypothesized genetic, epigenetic, developmental, and health/lifestyle-related factors.

Infertility and Cancer Risk

Multiple studies have investigated the prevalence of malignancies among infertile patients. One of the first associations explored was testicular germ cell tumors (TGCTs) in men with carcinoma in situ of the testis. Early results were confirmed by a meta-analysis of case-control studies that found a relative risk (RR) of 2.8 (95% CI: 1.16-6.72) for testis cancer among patients with infertility. Larger case-control studies using national registry data confirmed this evidence both in European and US populations. Richiardi et al. (2004) observed a lower risk of testicular cancer for men who have fathered children and Baker et al. (2005) observed that men with testis cancer were less likely to have fathered children compared to controls and they were more likely to be diagnosed with infertility. Others demonstrated an association between semen quality and testis cancer risk in a study of over 30,000 Danish men. In a multicenter study including 51,461 couples recruited from 15

centers in California showed that patients with male factor infertility had a threefold higher risk of testis cancer. Similar results were obtained in a recent retrospective study matching 20,443 men who underwent semen analysis with 20,443 fertile controls, showing that men with semen alterations had an increased risk of testis cancer with a hazard ratio (HR) of 3.3 as compared to controls.

Other male specific cancers have also been explored. The association between MFI and prostate cancer has conflicting data. While some authors reported a higher risk of prostate cancer, others failed to find a significantly higher risk of being diagnosed with prostate cancer using cohort studies. A meta-analysis including eleven studies showed a significantly lower risk of prostate cancer among childless men (using fatherhood as a surrogate for fertility). Importantly, a recent study by Boeri et al. observed that infertile men have higher prostate-specific antigen (PSA) values compared to fertile men. A meta-analysis reported that MFI is associated with an increased risk of both testicular (pooled RR: 2.03) and prostate cancer (pooled RR: 1.68) after comparing four studies (161,634 men) for testicular cancer and four studies (183,950 men) for prostate cancer. Besides urogenital malignancies, other types of cancer have been also associated with MFI; a US cohort demonstrated that patients with MFI have a 49% higher risk for being subsequently diagnosed with any cancer as compared to fertile men, including melanoma, prostate, testis, bladder, thyroid and hematological malignancies. Another study suggested a "dose response" relationship in MFI severity by demonstrating a higher risk of cancer in men with azoospermia compared to those with oligospermia.

Infertility and Metabolic Alterations

In addition to malignancies, other chronic diseases have linked semen quality and male reproductive function. The association between obesity and MFI has been observed in several studies. In a study investigating 26,303 patients by assessing the time to pregnancy, the risk of subfertility was 36% higher among obese compared to normal-weight men. These observations were confirmed by a larger European study in which the association between body mass index (BMI) and semen parameters has been also reported. In a meta-analysis including twenty-one studies, Sermondade et al. (2013) investigated the effect of BMI on sperm quality and demonstrated a significant association between BMI and

abnormal sperm count. Insulin resistance and diabetes mellitus (DM) may also affect male fertility. A meta-analysis including twelve observational studies evaluating the effect of DM on seminal parameters of patients screened for fertility, showed a 14% decrease in the percentage of motile cells among patients with DM compared to healthy controls. Finally, in a large prospective trial including thirty nine thousand subjects from the Danish national IVF register, 1.6% of men developed DM during follow-up. Importantly, the risk was significantly and progressively higher for patients with oligospermia, azoospermia, and aspermiacompared to fertile men. Indeed, the data suggest a common pathogenic background between diabetes and MFI.

In a recent study, slmost one in two primary infertile men presented with a triglycerides/glucose index (TyG) suggestive of insulin resistance showing worse clinical, hormonal, and semen parameters. In addition, abnormal lipid profiles have been observed among infertile men. The LIFE study (Eisenberg et al., 2015) showed that total cholesterol levels were associated with a decreased semen volume, while increased free cholesterol and phospholipid levels were associated with sperm structural and morphological abnormalities. Importantly, both male and female serum-free cholesterol concentrations were associated with increased time to pregnancy. Metabolic syndrome (MS), defined as the presence of three or more of the following risk factors: abdominal obesity, elevated fasting glucose, elevated triglycerides, low high-density lipoprotein (HDL) cholesterol, and elevated blood pressure, has been associated with MFI. Lotti et al. investigated the metabolic profile of 351 men with MS; of them, 27 (7.75%) were diagnosed with MS. Patients with MS had a lower rate of normal semen morphology. In addition, Ventimiglia et al. suggested a hormonal etiology and identified lower levels of total testosterone, sex-hormone-binding globulin, and inhibin-B in men with MS.

Infertility and Overall health

Both retrospective and longitudinal studies have investigated the role of MFI in overall men's health and mortality. Salonia et al. performed a case-control study including 344 European men with a diagnosis of MFI and 293 age-comparable fertile men using the Charlson Comorbidity Index (CCI) to objectively quantify the burden of patients' comorbidities. Infertile men had significantly higher CCI scores compared to fertile men. Interestingly, longitudinal studies

confirm the association between comorbidities and MFI. Data from the US show that among men with a diagnosis of MFI, the risk of developing subsequent comorbidities was significantly higher. Specifically, the risk of cardiovascular disease, diabetes, and also pathological habits such as alcohol abuse, was significantly higher in the infertile group. In addition, Latif et al. reported the hospitalization rates among 4712 infertile men was significantly associated with semen quality in a dose dependent fashion.

Infertility and Mortality

Male infertility has also been associated with mortality. In a large cohort of Danish men, a linear association btween semen quality (i.e. sperm motility and morphology) and the risk of death was identified. Importantly, the association was present for men with and without children. A US group evaluated nearly twelve thousand men and identifed an association with semen quality and mortality whereby men with at least two semen abnormalities had a two fold higher risk of death.

In contrast, a Swedish study did not identify an associaiton between male infertility and mortality. However, the most recent metaanalysis, does show that male infertility and impaired semen quality are associated with mortality risk. Recent studies suggest that the underlying mechanisms may include various developmental, hormonal, and environmental factors. For example, epigenetic alterations may lead to global changes in protien expression, which may impact spermatogenesis as well as other organ systems leading to the development of comorbidities affecting the health trajectory and thus male overall survival.

Summary

The recent literature supports the hypothesis that male infertility is a proxy of the overall male health status (both current and future). Several studies have linked MFI to an increased risk of testicular cancer. In addition, prostate cancer, melanoma, bladder, thyroid, and hematological malignancies have also been linked to a higher risk among infertile men. Large cohort studies have found a link between diabetes, metabolic abnormalities, and testicular function. Infertile men also appear to have a higher chance of acquiring cardiovascular disorders. A strong link has been discovered between semen anomalies and the overall burden of comorbidities,

as well as overall mortality. The fundamental pathophysiological relationship between infertility and other comorbidities is uncertain, but genetic, epigenetic, environmental, or developmental are leading theories.

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