CHAPTER

34

c0170

Sperm Physiology and Assessment of Spermatogenesis Kinetics *In Vivo*

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st0020

INTRODUCTION

p0010 It has been recently proposed that spermatogenesis and steroidogenesis are controlled by a master switch (gonadotropin releasing hormone (GnRH) pulse generator) that controls two separate and independent feedback systems: androgen production (LH-testosterone) and sperm production (FSH-inhibin) [1]. The testes require stimulation by the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), which are secreted in response to hypothalamic GnRH. Their action on germ cell development is affected by androgen and FSH receptors on Leydig and Sertoli cells, respectively. While FSH acts directly on the germinative epithelium, LH stimulates secretion of testosterone by the Leydig cells. Testosterone stimulates sperm production and virilization, and also feeds back the hypothalamus and pituitary to regulate GnRH secretion. FSH stimulates Sertoli cells to support spermatogenesis and to secrete inhibin B that negatively feedback FSH secretion.

p0015 When spermatozoa leave the testis, they are neither fully motile nor able to recognize or fertilize oocytes. Human spermatozoa must migrate through the epididymis and undergo a specific maturation process in order to become a functional gamete. The epididymis is a dynamic organ that promotes sperm maturation under the influence of androgens. It also provides a place for sperm storage and plays a role in the transport of the spermatozoa from the testis to the ejaculatory duct. In addition, the epididymis protects the male gametes from harmful substances and reabsorbs both fluids and products of sperm breakdown, thus enabling the sperm to fertilize the ovum and to contribute to the formation of a healthy embryo.

THE TESTIS: STRUCTURE AND FUNCTION

st0025

p0020 The human testis is an ovoid mass that lies within the scrotum. Since the process of sperm production is optimal at temperatures about 2 °C lower than that of core body temperature, the scrotum has mechanisms that facilitate the dissipation of heat, including a thin and wrinkled skin, minimal subcutaneous fat, sparse distribution of hair, a large number of sweat glands, and the cremaster and dartos muscles.

p0025 The testes in all mammals are paired encapsulated organs consisting of seminiferous tubules, which correspond to approximately 90% of testicular volume, separated by interstitial tissue. The testis weight and volume increases at puberty, reaching an average of 20 cm^3 in young men, and then decreases slightly with age [2]. Normal longitudinal length of the testis is approximately 4.5–5.1 cm. The average weight of the human testis is 15–19 g with a specific gravity of 1.038 g/mL [3]. The right testis is usually 10% larger than the left. Spermatogenesis probably decreases concomitantly to the decline in the overall testicular size [4].

p0030 The seminiferous tubules are long V-shaped tubules; both ends drain toward the central superior and posterior regions of the testis, the rete testis, which has a flat cuboidal epithelium. These cells appear to form a valve or plug that may prevent the passage of fluid from the rete into the tubule. The rete lies along the epididymal edge of the testis and coalesces in the superior portion of the testis, just anterior to the testicular vessels, to form 5–10 efferent ductules. The ductules leave the testis and travel a short distance to enter the head or caput region providing a connecting conduit for sperm transport to

Handbook of Fertility. http://dx.doi.org/10.1016/B978-0-12-800872-0.00034-2

ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170

the epididymis. The seminiferous tubules are arranged in about 300 lobules each containing one to four tubules. The rete testis is located in close proximity to the testicular artery that has part of its course on the surface of the testis. From the testis surface, the main artery branches turn back into the parenchyma and begin to ramify. The interstitial tissue fills up the spaces between the seminiferous tubules and contains all the blood and lymphatic vessels and nerves of the testicular parenchyma.

p0035 A layer of Sertoli cells coated by a lamina propria lines the seminiferous tubules, containing germ cells. The lamina propria consists of the basal membrane covered by peritubular cells (fibroblasts). The main component of the interstitial space is the Leydig or interstitial cells, but it also contains macrophages, lymphocytes, loose connective tissue, and neurovascular bundles.

p0040 The peritubular cells are distributed concentrically in layers around the seminiferous tubules, separated by collagen fibers. These cells produce extracellular matrix, connective tissue proteins (collagen, laminin, vimentin, fibronectin), and proteins related to cellular contractility such as smooth muscle myosin and actin. They also synthetize adhesion molecules including nerve growth factor (NGF) and monocyte chemoattractant protein 1 (MCP-1) [5]. Secretion of these factors is regulated by tubular necrosis factor-α (TNF-α), which in turn is produced by mast cells; as such, an interaction between peritubular and mast cells is suggested. It has also been shown that the number of mast cells increases in the testis in some infertility cases [6]. Peritubular cells have contractility properties that aid in the transport of sperm through the seminiferous tubules. Peritubular contractility is regulated by oxytocin, prostaglandins, androgens, and endothelin [7–9]. Endothelin is, in turn, modulated by the relaxant peptide adrenomedullin produced by Sertoli cells [7]. Peritubular cells also secrete insulin-like growth factor-1 (IGF-1) and cytokines that modulate the function of Sertoli cells, particularly the secretion of transferring, inhibin, and androgen-binding protein [8]. Due to the complex interactions between peritubular cells and other cellular elements, it has been suggested that these cells have a role in fertility. In fact, loss of contractility markers, tubular fibrosis, and sclerosis, as well as an increased number of mast cells, are seen in some derangements of spermatogenesis leading to subfertility [6,10,11]. Peritubular and interstitial fibrosis, in association with spermatogenic damage, have also been demonstrated in the testis of vasectomized men [12].

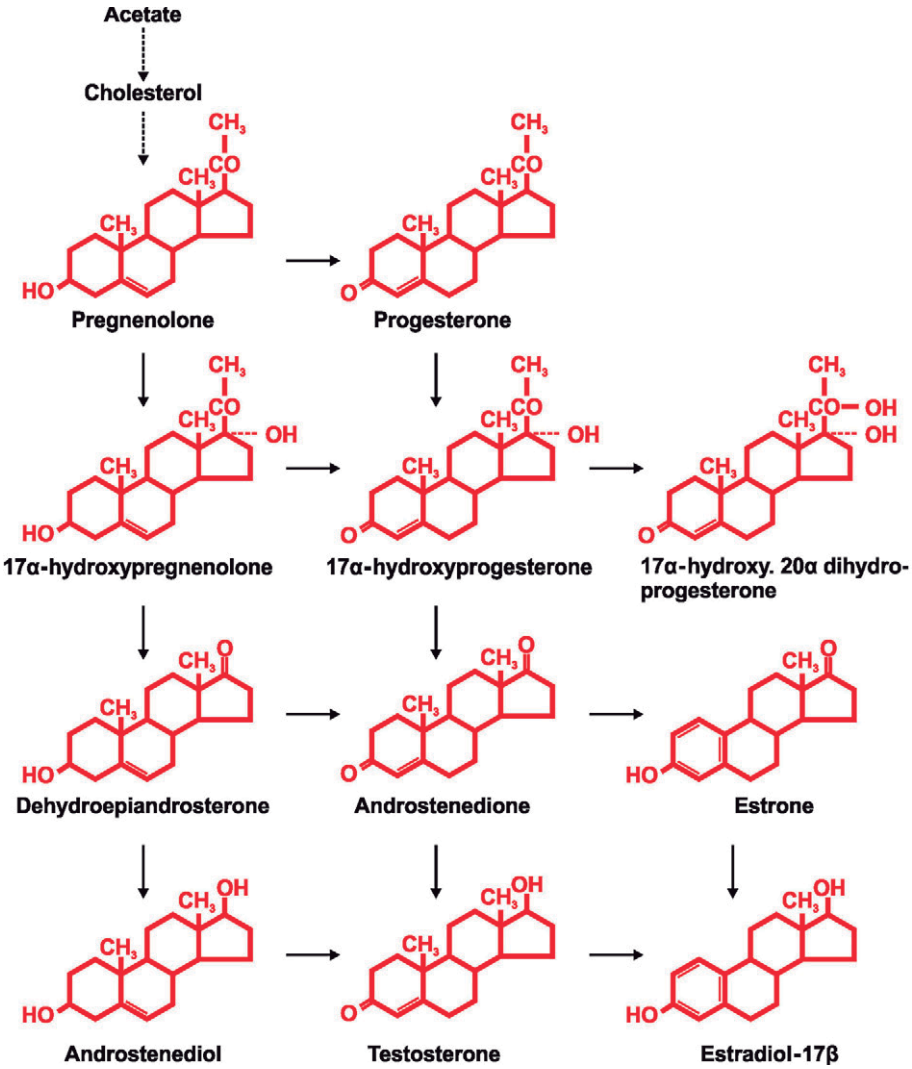
st0030 Androgen Production

p0045 The differentiation of Leydig cells is determined, at least in part, by peritubular and Sertoli cells, which secrete leukemia inhibitory factor (LIF), platelet-derived growth factor-α (PDGF-α), and other factors that trigger Leydig stem cells to proliferate and migrate into the interstitial compartment of the testis, where they differentiate in the so-called progenitor Leydig cells. After that, growth factors and hormones (LH, IGF-1, PDGF-α, and others) transform them into immature Leydig cells and, finally, into the adult Leydig cell population, which is primarily responsible for the production and secretion of testosterone [13]. Adult Leydig cells exhibit endoplasmatic reticulum and mitochondria, typical of a steroid-producing cell. The major substrate for androgen synthesis is cholesterol. Mitochondrial enzymes cytochrome P450SCC (side chain cleavage) or CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1) transform cholesterol into pregnenolone, a process of androgen synthesis limited by the availability of cholesterol substrate. In the so called delta-4 pathway, pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase, which in turn is converted to 17α-hydroxyprogesterone and androstenedione by 17α-hydroxylase or CYP17A; androstenedione is finally converted to testosterone by cytochrome P450c17 (Fig. 34.1). In the delta-5 pathway, pregnenolone is hydroxylated to 17α-hydroxypregnenolone and dehydroepiandrosterone by 17α-hydroxylase or CYP17A, which in turn are converted to androstenediol by cytochrome P450c17; finally, androstenediol is transformed into testosterone by 3β-hydroxysteroid dehydrogenase. Testosterone can be converted to estradiol by aromatase or to dihydrotestosterone by 5α-reductase. LH stimulates the transcription of genes that encode the enzymes involved in the steroidogenic pathways to testosterone.

Sperm Production st0035

p0050 Sertoli cells form the structure of the seminiferous tubules; their base rest on the basement membrane and their apex is oriented toward the lumen of the tubule (Fig. 34.2). Tight junctions between adjacent cells create a basal compartment that acts as a blood–testis barrier. Spermatogonia and early preleptotene primary spermatocytes are enclosed in the basal compartment while spermatocytes and spermatids are confined to the adluminal compartment [14]. Spermatocytes and spermatids first appear at puberty and therefore after the development of the immune system. The blood–testis barrier separates spermatocytes and spermatids from the immune system, thus avoiding the formation of autoantibodies. A continuous remodeling of the tight junctions, mediated by proteases and protease inhibitors self-secreted by Sertoli cells, occurs as the germ cells are transferred from the basal to the adluminal compartment [15]. Sertoli cells synthesize and secrete a large number of proteins, under the influence of testosterone and FSH [16,17], such as transport proteins (transferrin, ceruloplasmin, androgen binding protein), proteins involved

ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170



f0010 **FIGURE 34.1** Steroidogenic pathways to testosterone production. *Reprinted from Ref. [102], with permission from Jaypee Brothers Medical Publisher.*

in tight junction remodeling (cadherins, connexins, laminins), and regulatory proteins (antimullerian hormone, seminiferous grown factor), which are crucial for their interaction with germ cells. The functions of Sertoli cells include the regulation of spermatogenesis (by providing support and nutrition to germ cells) and the release of spermatids and spermiation.

p0055 The germinative cells are enclosed within the compartments created by the Sertoli cells and the tubular lumen (Fig. 34.3). Three types of spermatogonia are found in the basal compartment: (1) type A dark (considered as testicular stem cells), (2) type A pale (replicate by mitosis), and (3) type B (the cell type that will progress into meiosis) [1,18]. Cohorts of type B spermatogonia initiate meiosis by entering the leptotene stage of the first meiotic prophase. Meiosis is initiated after mitotic proliferation of spermatogonia by DNA synthesis that accomplishes precise replication of each chromosome to form two chromatids. Thus, the DNA content doubles but the number of chromosomes remains the same, that is, diploid. These cells are the primary spermatocytes; they progressively show the nuclear features that identify meiosis I stages of leptotene, zygotene, pachytene, and diplotene. During meiosis I, homologous chromosome pair, forming bivalents, and undergo reciprocal recombination, resulting in a new combination of gene alleles. The first meiotic division is reductional, separating the members of each homologous pair and reducing the chromosome number from 2N to 1N. The result is two haploid cells, secondary spermatocytes, each with 23 chromosomes, but with each chromosome still comprised of two chromatids. The meiosis II is an equational division that separates the chromatids to separate cells, each containing the haploid number of chromosome and DNA content. The products of these meiotic divisions are four spermatids (Fig. 34.4).

p0060 The arrangement of germ cells along the basal compartment of the seminiferous tubules differs between

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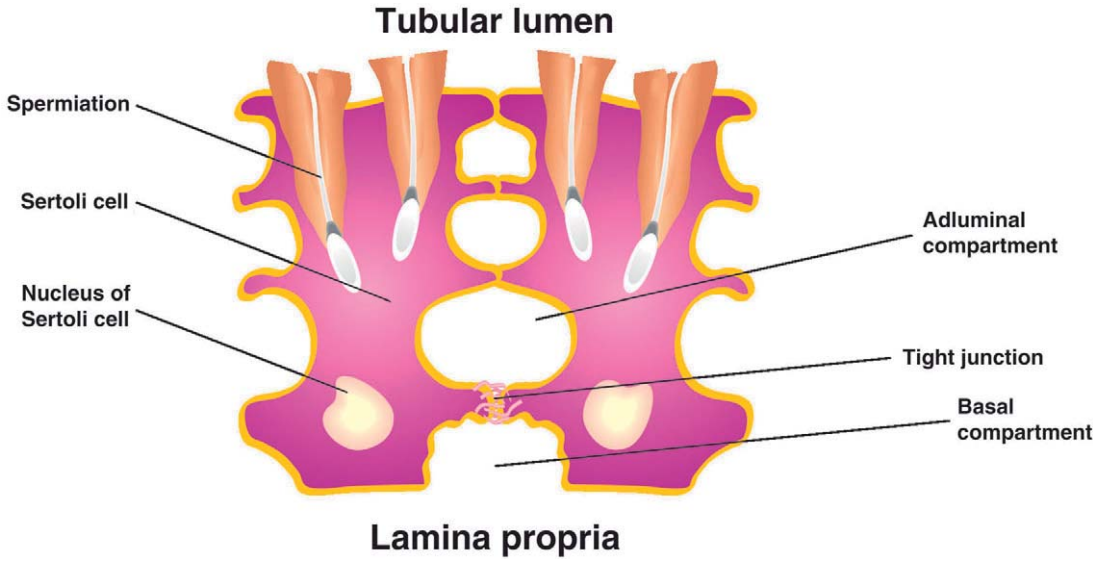
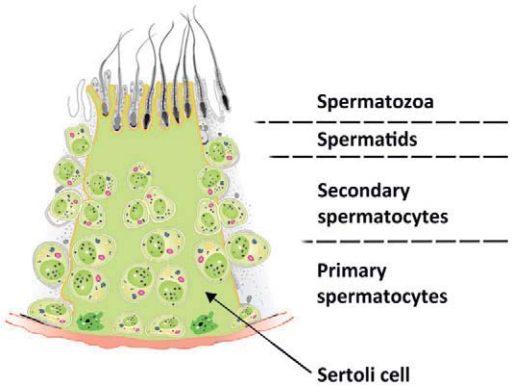


f0015 **FIGURE 34.2** Schematic representation of Sertoli cells and their relation to the compartments that enclose the germ cells. *Reprinted from Ref. [102], with permission from Jaypee Brothers Medical Publisher.*

species [1]. While in the mouse all germ cells in a cross-section of a seminiferous tubule are in developmental synchrony, the situation in man is different as germ cells at different developmental stages can be identified in each tubular cross-section. In addition, the longitudinal extension of the premeiotic germ cell cohorts attached to the basement membrane area also differs between species. In man the cohorts of germ cells are much shorter than in mice (Fig. 34.5).

p0065 When meiosis is completed, the haploid round spermatids are conjoined in a syncytium as they initiate the final differentiation stage of spermatogenesis, termed spermiogenesis. It involves no cell division, representing a complex series of cytological changes leading to the transformation of the round spermatids to spermatozoa. In humans, there are eight different stages ($S_{a\text{-}1}$, $S_{a\text{-}2}$, $S_{b\text{-}1}$, $S_{b\text{-}2}$, $S_{c\text{-}1}$, $S_{c\text{-}2}$, $S_{d\text{-}1}$, and $S_{d\text{-}2}$) involved in the maturation of spermatids to spermatozoa. This process includes (1) changes in the position of the nucleus from a central to an eccentric location, together with reduction in the nuclear size and condensation of the nuclear DNA (chromatin compaction); (2) formation of the acrosome from the Golgi complex, which interposes between the nucleus and the cell membrane; (3) tail formation from a pair of centrioles lying adjacent to the Golgi complex and the aggregation of mitochondria; (4) elimination of most of the cytoplasm, which are phagocytosed by Sertoli cells. Once formed, sperm is released into the tubular lumen (spermiation). In summary, spermatogenesis and spermiogenesis involve a series of extremely intricate processes of cell differentiation, which begin in the basal compartment and end in the apical compartment, and that result in the production of highly specialized spermatozoa with fully compacted chromatin.



f0020 **FIGURE 34.3** Schematic representation of a histologic cross-section of a single seminiferous tubule depicting its cell components.

Spermatozoon Structure

st0040

p0070 A morphologically normal sperm cell is about 45–50 μm in length and consists of a head and tail (Fig. 34.6). A morphologically normal head is smooth and symmetrically oval in shape with a broad base and tapering apex. The sperm head measures between 4.0–5.5 μm in length and 2.5–3.5 μm in width, with a length-to-width ratio of between 1.50 and 1.70 [19]. The head is the most important part of the mature male gamete as it contains a nucleus, which is composed of packed chromosomal paternal genetic material (mostly DNA) containing 23 chromosomes. The nucleus comprises about 65% of the head, but like most somatic cells, lacks a large cytoplasm in contrast to somatic cells [20]. The head also contains a well-defined acrosome region, a cap-like covering of the anterior two thirds of the head (40–70% of the apex). The acrosome is represented by the Golgi complex and contains a number

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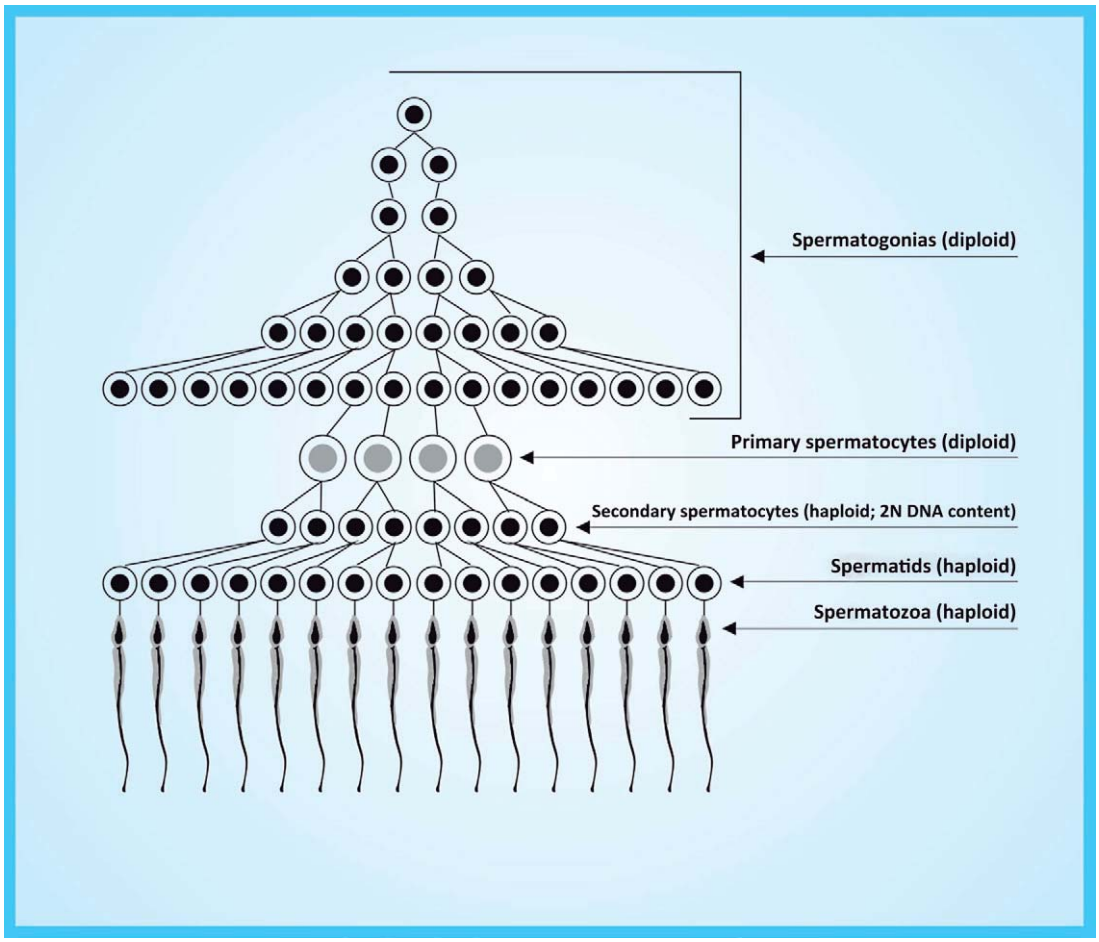

f0025 **FIGURE 34.4** Schematic representation of cell division stages during spermatogenesis.

of hydrolytic enzymes, such as hyaluronidase and acrosin, which are required for fertilization [20].

p0075 The sperm tail measures 40–50 μm in length (nearly 10 times the length of the head) and between 0.4 μm and 0.5 μm in diameter. It is divided into the midpiece, principal piece, and endpiece. The midpiece supports the head at exactly the center position. It is slender (maximum width of 1 μm), yet thicker than the rest of the tail and measures between 7.0 μm and 8.0 μm in length. The midpiece consists of tightly packed mitochondria surrounded by a sheath. The mitochondria in the midpiece supply energy in the form of ATP for tail movement. The principal piece is the longest part of the tail and comprises most of the propellant machinery. The normal tail has a well-defined endpiece, without any coiling or abnormal bending.

st0045 ## THE EPIDIDYMIS: STRUCTURE AND FUNCTION

p0080 The epididymis is a single highly convoluted duct extending from the anterior to the posterior pole of the testis. It is closely attached to the testis surface by connective tissue and the tunica vaginalis, which surrounds the testis and the epididymis except for its posterior aspect. The posterior surface is attached to the scrotum and spermatic cord by a fibrofatty connective tissue [21]. The epididymis consists of the ductuli efferentes and the ducts epididymis. Between 10 and 15 ductuli efferentes arise from the rete testis. These tubules come together to form the epididymal duct that varies from 3 m to 4 m [22]. This convoluted tube folds repeatedly upon itself and forms the main bulk of the organ. The epididymis is conventionally divided in the caput, the corpus, and cauda. An alternative subdivision has been proposed by Glover and Nicander [23], which is based on histologic and functional criteria, and it divides the epididymis into three regions: initial, middle, and terminal segments. The initial and middle are primarily concerned with sperm maturation, and the terminal segment coincides with the region where mature sperm are stored before ejaculation [24,25]. The initial segment comprises the region where the ductuli efferentes empty, while the terminal portion disappears into the epididymal fat making it appear that the epididymis has a minimal cauda region. In fact, the still-coiled human epididymis is 10–12 cm long before

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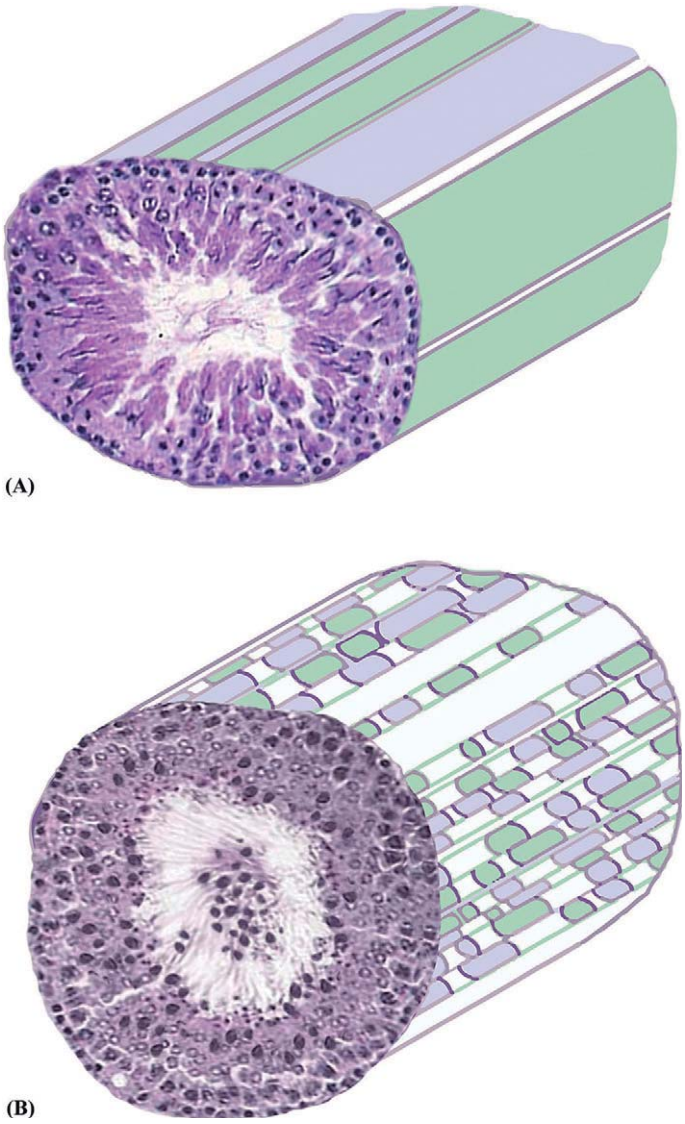

f0030 **FIGURE 34.5** Schematic arrangement of germ cells along the basement membrane of the seminiferous tubules. (A) The mouse is an example of a species in which a single seminiferous epithelial stage arrangement is seen at a given cross-section of a seminiferous tubule. (B) In man, there is a mixed seminiferous epithelial stages at a given cross-section of a seminiferous tubule. For both species the longitudinal extension of the premeiotic germ cell cohorts attached to the basement membrane are depicted. The cohorts of germ cells are larger in humans compared with mice. *Adapted from Ref. [1], copyright 2014, with permission from Elsevier.*

becoming the convoluted vas, and the convoluted vas extends for approximately another 7–8 cm [26].

p0085 The epididymal epithelium is composed of several cell types that include the principal, basal, apical, halo, clear, and narrow cells. The distribution of these cells varies in number and size at different points along the epididymal duct [26,27]. The primary cell type throughout the tubule is the principal cell, which constitutes approximately 80% of the epithelium and is, by far, the most studied since it is responsible for the bulk of the proteins that are secreted into the lumen [28]. The infranuclear compartments of the principal cells are rich in rough endoplasmic reticulum, and the supranuclear of these cells have numerous mitochondria and highly developed Golgi complexes. The principal cells are responsible for secretion of carnitine, glycerylphosphorylcholine and sialic acid, inositol, and a variety of glycoproteins [29]. Narrow, apical, and clear cells contain the vacuolar H^+-ATPase and secrete protons into the lumen and thus participate in its acidification [30,31]. Clear cells are endocytic cells and may be responsible for clearance of proteins from the epididymal lumen. Basal cells do not access the luminal compartment and are in close association with the overlying principal cells, as indicated by the presence of basal cell cytoplasmic extensions between principal cells, and thus may regulate its functions [32,33]. Halo cells appear to be the primary immune cell in the epididymis, while apical cells may

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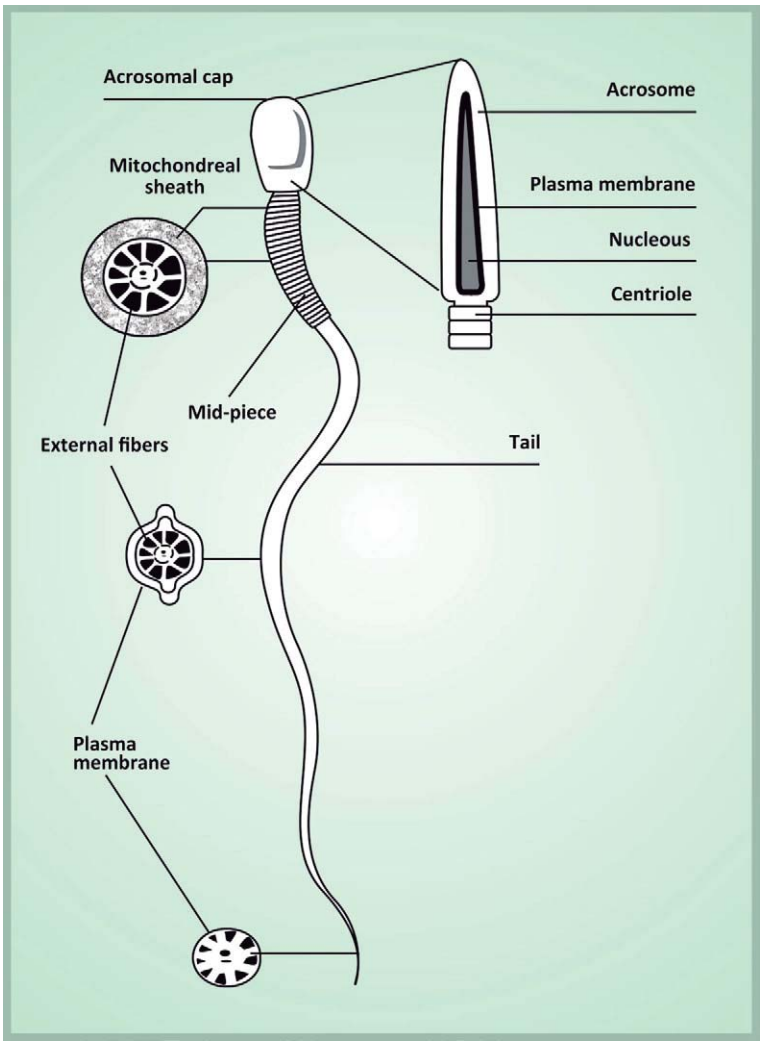

f0035 **FIGURE 34.6** Schematic representation of a mature human spermatozoon showing its components, the head, midpiece, and tail.

also endocytose luminal components. The most detailed study of the epithelia and the tubule organization in the human epididymis came from Yeung et al. [34]. The authors described at least seven types of tubules, connected by at least eight types of junctions to form a network, each one characterized by a different epithelium. The differences in the cellular architecture are primarily due to the functional roles of each epithelium within each epididymal region. In the proximal region there is considerable absorption of water, hence the epithelium takes on the classical appearance of a water-absorbing surface with long stereocilia and many mitochondria in the basal aspects. The cells at the distal epididymis are much smaller and are specialized in removing cellular debris.

st0050 ## Sperm Maturation

p0090 Spermatozoa mature during epididymal transit and acquire functional competence. Although the mechanisms by which the epididymis perform its functions of sperm maturation, transport, and storage are not completely understood, it is believed that these functions are affected by the cells and fluid milieu within the epididymal lumen [26]. During their development, spermatozoa are continually bathed in fluid secretions provided by the seminiferous tubules and epididymal ducts [27]. Microanalytic studies revealed that several biochemical changes fundamental for sperm maturation and survival occur in the epididymal duct intraluminal fluid [35,36]. The fluid in the proximal epididymis is quite acidic with pH values within the 6.5 range; it increases to approximately 6.8 in the distal region. A variety of substances are secreted by the epididymis and these substances may influence sperm maturation. The epididymal lumen contains the most complex fluid found in any exocrine gland. It results from the continuous changes in composition as well as the presence of components, such as L-carnitine, glutamate, inositol, sialic acid, taurine, glycerophosphorylcholine, and lactate in unusually high concentrations for reasons not yet known [28]. Epididymal tubule segments

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represent unique physiological compartments, each one possessing distinctive gene expression profiles within the epithelium that dictate segment-specific secretion of proteins into the luminal fluid that affect sperm maturation.

p0095 Sperm maturation involves alterations in the plasma membrane and chromatin condensation and stabilization [37]. Sperm remodeling also includes changes in the dimension and appearance of the acrosome and nucleus, migration of the cytoplasmic droplet along the tail, as well as structural changes in intracellular organelles [38]. Spatially separated lipids and proteins are reorganized during maturation possibly allowing the formation of signaling complexes critical for fertilization. These changes are promoted by the fluid microenvironment within the epididymis and ensure that sperm can transverse the female reproductive tract, and after capacitation, fertilize the oocyte [39]. The epididymal fluid is hyperosmotic and the major constituents are L-carnitine, glutamate, inositol, sialic acid, taurine, glycerophosphorylcholine, and lactate. Concentrations of these substances can reach from 20 mM to 90 mM depending upon the epididymal region. Sodium, potassium, bicarbonate, and chloride are also present in the luminal fluid [39]. The organic substances, the electrolytes, and the enzymes may be involved in the acquisition of motility, in sperm and epididymal cell metabolism, and in the osmoregulation for sperm and epididymal epithelium cells. Several proteins, such as albumin, transferrin, immobilin, clusterin (SGP-2), metalloproteins, and proenkephalin, are also found within the epididymal lumen and are claimed to be associated with sperm maturation [22].

st0055 ### Sperm Transport

p0100 Spermatozoa released from the Sertoli cells into seminiferous tubule lumen transit approximately 6 m in the reproductive tract before they leave the urethral meatus [26]. These cells move in part by hydrostatic pressure originating from fluids secreted in the seminiferous tubules and by tubular peristaltic-like contractions. Contractions of the tunica albuginea play a role in the generation of positive fluid pressure onto the head of the epididymis [22,23]. Transport through the proximal epididymis is due to peristaltic contractions of the smooth muscles surrounding the epididymal duct. The mechanisms responsible for driving the spermatozoa through the epididymal lumen include fluid currents established by action of cilia along the walls of the efferent ducts, hydrostatic pressure gradients, and spontaneous rhythmic contractions of the contractile cells surrounding the epididymal duct [23]. Adrenergic and cholinergic mechanisms and vasopressin have been proposed as regulating factors for the epididymal duct peristaltic activity. Transport rates are estimated to be more rapid in the efferent ducts and proximal epididymis where the fluid is non-viscous and water is rapidly absorbed from the luminal compartment [22].

SPERM FUNCTION st0060

p0105 *In vivo*, ejaculated sperm from all eutherian mammals are unable to fertilize until they have undergone capacitation, which allows the acrosome reaction (AR) to take place when spermatozoa approach or contact the oocyte [40–43]. Capacitation is a time-dependent phenomenon, with the absolute time course being species-specific [44]. It prepares the sperm to undergo the AR with the accompanying release of lytic enzymes and exposure of membrane receptors, which are required for sperm penetration through the zona pellucida (ZP) and fusion with the oolema [44]. Sperm transport through the female genital tract can occur quite rapidly (times as short as 15–30 min have been reported in humans), whereas capacitation may take from 3 h to 24 h [44]. It is speculated; therefore, that capacitation is not completed until after the spermatozoa have entered the cumulus oophorus. This delay is physiologically beneficial because spermatozoa do not respond to AR-inducing signals until they approach the ZP, preventing premature ARs that lead ultimately to the sperm's inability to penetrate the egg vestments [44,45]. Sperm capacitation is a postejaculatory modification of the sperm plasma membrane, which involves mobilization and/or removal of surface components, such as glycoproteins, decapacitation factor, acrosome-stabilizing factor, and acrosin inhibitor. Sperm capacitation involves major biochemical and biophysical changes in the membrane complex and energy metabolism. The presence of high concentrations of cholesterol in the seminal plasma, which maintains a high cholesterol concentration in sperm membranes, seems to be the most important factor for inhibiting capacitation [46]. Capacitation is associated with increased membrane fluidity caused by the removal of cholesterol from sperm plasma membrane via sterol acceptors present in the female tract secretions [47,48]. A marked change in sperm motility, named hyperactivation, is also associated with capacitation. Hyperactivated spermatozoa exhibit an extremely vigorous but nonprogressive motility pattern, as a result of a Ca^{2+} influx, that causes increased flagellar curvature [49] and hence extreme lateral movement of the sperm head [47]. Proteasome participates in activating calcium channels that also leads to increased membrane fluidity and permeability [50–53]. These events are followed by, or occur simultaneously with, (1) a decrease in net surface charge, (2) devoided area of intramembrane protein and sterols, and (3) increased concentrations of anionic phospholipids [53,54]. Hyperactivated motility is essential for sperm penetration into the intact oocyte–cumulus complexes both *in vitro* and *in vivo* [55,56].

ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170

p0110 The spermatozoon binds to the ZP with its intact plasma membrane after penetrating into the cumulus oophorus. Sperm binding occurs via specific receptors to ZP glycoproteins located over the anterior sperm head [57]. Glycosylation of ZP glycoproteins is an important aspect of sperm–ZP interaction. It is believed that human ZP glycoprotein-3 (ZP3) has a central role in initiating the AR [58]; however, it has been recently demonstrated that human ZP1 and ZP4 are also implicated in the process [59,60]. The AR is a stimulus-secretion coupled exocytotic event in which the acrosome fuses with the overlying plasma membrane [61,62]. The multiple fusions between the outer acrosomal membrane and the plasma membrane result in the release of hydrolytic enzymes (mostly acrosin) and in the exposure of new membrane domains, both of which are essential if fertilization is to proceed further. The hydrolytic enzymes released from the acrosome digest the ZP, allowing the spermatozoa to penetrate the oocyte [57]. The AR seems to be physiologically induced by natural stimulants such as the follicular fluid (FF), progestin, progesterone, and hydroxyprogesterone [54]. Follicular fluid and cumulus cells have protein-bound progesterone that has been identified as one of the most important acrosome-reaction-inducing agents [62,63]. Follicular fluid stimulates the AR in a dose-dependent manner [63,64]. A link between locally produced estradiol by ejaculated spermatozoa, AR, and sperm capacitation was described [65]. Moreover, evidence indicates that environmental estrogens can significantly stimulate mammalian sperm capacitation and AR [66]. The AR is probably initiated when ligands produced by the oocyte bind to the receptors on the spermatozoa. This signal is transduced intracellularly via second messengers, ultimately leading to exocytosis [67]. A number of second messenger pathways have been identified in human spermatozoa, including those that result in the activation of cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and phospholipid-dependent protein kinases [53,68–70]. These kinases are called, respectively, protein kinase A, protein kinase G, and protein kinase C. It is possible that these pathways interact to assure an optimal response at the correct place and time during the fertilization process. Although the concentration of cGMP in ejaculated human semen is almost seven times lower than cAMP, it is speculated that both nucleotides have a similar role in the AR since their dependent protein kinases are closely related proteins [71–73]. The AR can also be induced by artificial stimulants that cause and increase in sperm intracellular calcium [53,74].

p0115 Acrosome integrity is crucial for normal fertilization. A high proportion of sperm with intact acrosomes is seen in ejaculates of normal men. In such individuals, ~5–20% sperm cells may exhibit spontaneous ARs that are of no clinical significance [74]. Conversely, several abnormal conditions affecting the sperm acrosome may lead to decreased fertilization ability. Acrosomeless round-headed spermatozoa (globozoospermic spermatozoa) are unable to fertilize the oocytes, and increased percentages of morphologically abnormal acrosomes were related to fertilization failure in assisted reproductive technology (ART) using conventional *in vitro* fertilization (IVF) [75]. The AR is a time-dependent phenomenon that cannot take place prematurely or too late [76]. Premature AR and the inability of the spermatozoa to release the acrosomal contents in response to proper stimuli (AR insufficiency) have been associated with idiopathic male infertility [77]. Although the cause of premature AR is unknown, the premature (stimulus-independent) initiation of acrosomal exocytosis seems to be related to a perturbation of the plasma membrane stability. In this situation, the AR may not involve a premature activation of the receptor-mediated process, but rather reflect an inherent fragility of the sperm membrane, leading to a receptor-independent acrosomal loss [78]. Antisperm antibodies (ASA) may adversely affect the ability of sperm to undergo capacitation and AR [79]. Chang et al. [80] reported a reduction in fertilization rate either by IgG directly bound to sperm or IgM present in female serum. The combination of IgG and IgA may have a synergistic negative effect on fertilization [81–84]. Toxic substances to sperm can also impact the AR. High concentrations of dietary phytochemicals, such as genistein, isoflavone, and β-lapachone, were shown to suppress the AR in a dose- and time-dependent manner in the rat model [46]. Inhibition of AR by genistein seems to involve the protein kinase C pathway while β-lapachone has a direct cytotoxic effect on sperm cell membrane. It is suggested that genistein and β-lapachone may impact male fertility via AR suppression in high doses and AR induction in low doses [85]. Calcium channel blockers may also interfere with the AR exocytotic event. Sperm incubation with different blockers, such as trifluoperazine (calmodulin inhibitor), verapamil (Ca^{2+} channel inhibitor), and nifedipine (voltage-dependent Ca^{2+} channel inhibitor) significantly reduced the ability of hamster sperm to undergo the AR [86].

p0120 Recent publications on AR focused on the biochemical and functional aspects of sperm–oocyte fusion. It has been demonstrated that in humans, ZP1 in addition to ZP3 and ZP4 binds to capacitated spermatozoa and induces acrosomal exocytosis [59,87–89]. The ZP3-induced AR involves the activation of T-type voltage-operated calcium channels (VOCCs), whereas ZP1- and ZP4-induced ARs involve T- and L-type VOCCs. Chiu et al. [90] reported that glycodelin-A, a glycoprotein present in the female reproductive tract, sensitizes spermatozoa to the ZP-induced AR in a glycosylation-specific mechanism involving the activation of the adenylyl cyclase/PKA

ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170

pathway, suppression of extracellular signal-regulated kinase activation, and upregulation of ZP-induced calcium influx. It is therefore suggested that glycodelin-A may be important *in vivo* to ensure full responsiveness of human spermatozoa to the ZP.

st0065

ASSESSMENT OF SPERMATOGENESIS KINETICS *IN VIVO*

st0070

The Past

p0125 Among the most important discoveries in the past century in the field of spermatogenesis was the identification of the seminiferous epithelial cycle in mammals [91,92]. The experimental approach initially used in animals to determine the duration of the germinative cycle consisted of an analysis of the rate of disappearance of germ cells from the seminiferous tubules after testis irradiation. When administered in proper doses, X-rays destroy a large percentage of spermatogonia, which results in a progressive disappearance of spermatocytes and spermatids from the seminiferous tubules. Obviously, this method could not be applied to humans.

p0130 With the advent of radioactive tracers and the development of radioautography, it became possible to determine the duration of the cycle in intact animals. This method involved radiographic analysis of sequential biopsies after the testes have been injected with tracers. Tritiated thyamidine, which is selectively incorporated in the nuclei of cells preparing for mitosis or meiosis, became the label of choice and was extensively used to time the cycle. Quantitative analysis of the tubular cross-sections containing labeled cells yielded consistent results for the duration of the cycle.

p0135 In 1963, a breakthrough study was conducted by Heller and Clermont in which a group of seven men who had undergone vasectomy received an intratesticular injection of tritiated thiamidine, and was followed with serial testicular biopsies [93]. Based on these data, the production of spermatocytes from spermatogonia was estimated to be 16 days, and the duration of the three phases of spermatogenesis, namely, (1) the proliferation of spermatogonia to give rise to diploid spermatocytes; (2) meiotic division, which gives rise to spermatids; and (3) cytological transformation, which leads to mature spermatozoa, was estimated to be 64 days, a value exclusive of epididymal transit time. The human epididymal transit time has been estimated to be 5.5 days, and this estimate is derived from early mammalian studies mainly involving pigs [94]. Our knowledge that human spermatogenesis requires approximately 3 months to complete comes from these data, and they have been used for the last five decades to guide the time lines proposed in the clinical management of male infertility [94].

p0140 Due to its toxicity and invasiveness, the aforementioned studies could never be repeated in men. Nevertheless, their findings have helped investigators to understand the different cellular events that occur during spermatogenesis. For instance, although a clear-cut cycle of the seminiferous epithelium was difficult to describe in humans due to an apparent mixing of germ cells, the analysis of serial sections in well-fixed biopsies revealed typical cell associations into six stages. A mixing of germ cells at the interface of adjacent cell associations was present, including a frequent absence of one or more germ cell generations. Of note, the number of germ cells at the same developmental stage was relatively small, and occupied restricted tubular areas [92].

The Present

st0075

p0145 A noninvasive and nontoxic method for accurately measuring spermatogenesis kinetics *in vivo* would be ideal for basic research and clinical applications. Such a method might allow the assessment of the effects of infertility treatments in the form of surgery or medication and the effects of environmental agents or gonadotoxins on testicular function.

p0150 In 2006, Misell et al. described a nontoxic and noninvasive method for measuring spermatogenesis *in vivo* [95]. It involved the use of a stable isotope labeling with 70% enriched heavy water (^2H_2O) and analysis of DNA isotopic enrichment in ejaculated sperm by gas chromatography/mass spectrometry (GC/MS). The authors also characterized the kinetics of human spermatogenesis *in vivo* in a group of healthy men with normal sperm production.

p0155 Briefly, the method described by Misell et al. involved the daily intake of ^2H_2O for 3 weeks in order to achieve and maintain a body water enrichment of approximately 1.5%. This level of body water enrichment has been previously shown to allow adequate label incorporation for subsequent analysis of DNA synthesis (Fig. 34.7) [96–98]. In their experiment, a total of 11 healthy men with normal sperm concentrations ingested deuterated (heavy) water (^2H_2O) daily and semen samples were collected every 2 weeks for up to 90 days. Label incorporation into sperm DNA was quantified by gas chromatography/mass spectrometry, allowing calculation of the percent of new cells in ejaculates. The overall mean time to detection of labeled sperm in the ejaculate was 64 ± 8 days (Fig. 34.8).

p0160 The aforementioned study represents the first time in five decades that a noninvasive direct kinematic measurement of spermatogenesis *in vivo* has been done in humans. In contrast to the study of Heller and Clermont, in which spermatogenesis was estimated to take 64 days excluding epididymal transit time, Misell et al. showed that spermatogenesis duration was much shorter. The authors showed that the appearance of new sperm in

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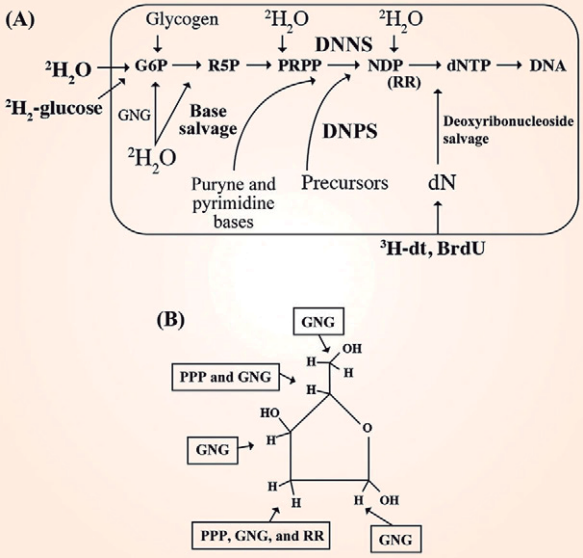
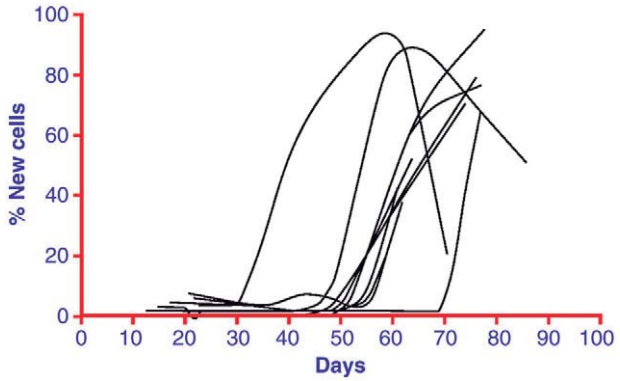


f0040 **FIGURE 34.7** (A) Labeling pathways for measuring DNA synthesis and thus cell proliferation. (B) Sites of 2H incorporation from ^2H_2O into C─H bonds of dR in replicating DNA. GNG, gluconeogenesis/glycolysis; PPP, pentose–phosphatase pathway; RR, ribonucleotide reductase; DNPS, de novo purine/pyrimidine synthesis pathway; DNNS, de novo nucleotide synthesis pathway. *Reprinted from Ref. [98], copyright (2002), National Academy of Sciences, USA.*



f0045 **FIGURE 34.8** Spermatocyte labeling curves for 11 subjects with normal semen analyses. Cases were labeled with 50 mL 70% deuterated (heavy) water (^2H_2O) twice daily for 3 weeks. Semen samples were collected every 2 weeks for 90 days from start on ^2H_2O. Spermatocyte DNA enrichment was measured by gas chromatography/mass spectrometry and compared to that of fully turned over cell (monocyte) to calculate the percentage of new cells present. A considerable interindividual variability between normal subjects was observed. *Reprinted from Ref. [96], copyright 2006, with permission from Elsevier.*

ejaculates of normal men occurred at a mean of 64 days, but this value included epididymal transit time.

p0165 The time to new sperm in the ejaculates was 42–76 days, as observed by Misell et al., represents a significant interindividual variation, which contradicts the current belief that spermatogenesis duration is fixed among individuals. While in one subject the time lag was 42 days to detect greater than 33% of new sperm at this time point, in all others it took at least 60 days. All subjects achieved greater than 70% new sperm in the ejaculate by day 90, but plateau labeling was not attained in most, suggesting rapid washout of old sperm in the epididymal reservoir. Although these findings can be related to a variation in spermatogenesis per se, it could also be possible that they were influenced by epididymal transit time, since a separate analysis of spermatogenesis and epididymal transit duration was not possible using their methods. Indeed, it has been speculated that epididymal transit time varies due to the rate of passage through the epididymis cauda, which in turn are influenced by ejaculatory frequency [99]. Also, it has been suggested that men with high testicular sperm production have shorter epididymal transit time than men with lower testicular sperm output [100]. This difference might be explained by a direct association between the production of sperm and fluid, since testes that produce more sperm also produce more fluid, so the movement of spermatozoa along the epididymal duct could be more rapid.

p0170 The Misell et al. data also suggested that in normal men sperm released from the seminiferous epithelium enter the epididymis in a coordinated manner with little mixing of old and new sperm before subsequent ejaculation. It is a novel concept since it had been suggested that because of mixing, in any segment of the epididymal duct, the population of sperm would be heterogeneous in age and biological status. Their kinetic data showed a sharp increase and subsequent steep decrease in sperm enrichment in the men with complete labeling curves, thus indicating that sperm age is not heterogeneous (Fig. 34.8). If significant mixing of young and old sperm had occurred, the slope of the delabeling curve would have been far more gradual. These kinetic data suggest that the epididymal reservoir is purged of old sperm fairly rapidly and completely in normal men.

CONCLUSIONS

st0080

p0175 Spermatogenesis is a highly organized and complex sequence of differentiation events that yields genetically distinct male gametes for fertilization. Sperm production is a continuous process, initiated at puberty and continuing throughout life, which occurs in the seminiferous tubules within an immune privileged site. Spermatozoa released from the seminiferous tubules into the epididymis undergo post-testicular maturation. Before fertilization can occur, spermatozoa must undergo further biochemical changes via capacitation and the AR, both of which occur after ejaculation. Recent knowledge originated from a novel direct measurement of human spermatogenesis kinetics *in vivo* indicates that the entire sperm production process is shorter than previously believed. Based on this new method involving a stable isotope labeling with enriched heavy water

ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170

and analysis of DNA isotopic enrichment in ejaculated sperm by gas chromatography/mass spectrometry, it has been also suggested that there is a large individual biological variability in the duration of spermatogenesis. This method may become a novel tool for characterizing the relationship between spermatogenesis and semen quality in male infertility, including the measurement of the effects of gonadotoxic exposure as well as medical and surgical interventions. bi0010

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ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170

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Non-Print Items

Abstract

Male fertility in humans depends on the continuous daily production of millions of spermatozoa. Spermatogenesis is a sequence of highly intricate stages by which an undifferentiated diploid spermatogonium matures into a specialized, genetically unique haploid spermatozoon. Spermatozoa leaving the Sertoli cells enter the epididymis for final maturation. This process requires a very elaborated interplay of autocrine, paracrine, and endocrine factors. Classically, the duration of spermatogenesis from the differentiation of pale spermatogonia to the ejaculation of mature spermatozoa has been estimated to be around 3 months. Recent data originated from a novel noninvasive method for direct measurement of human spermatogenesis kinetics *in vivo*, which applies isotope labeling with enriched heavy water and analysis of DNA isotopic enrichment in ejaculated sperm by gas chromatography/mass spectrometry physiology, indicate that the appearance of new sperm in the ejaculate occur much faster, at a mean time of approximately 2 months. A significant interindividual biological variability also seems to exist as the time lag for the completion of a full spermatogenic cycle and have new sperm in the ejaculate ranges from 42 to 76 days. This new knowledge on *in vivo* spermatogenesis kinetics is likely to change the time lines proposed for improvement or recovery in countless infertile couples undergoing male infertility treatment worldwide.

Keywords: epididymis; male infertility; physiology; sperm; sperm kinetics; spermatogenesis; testis

ISBN: 978-0-12-800872-0; PII: B978-0-12-800872-0.00034-2; Author: WATSONENGLISH; Document ID: 00034; Chapter ID: c0170