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Spermatogenesis in Humans and Its affecting factors

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Abstract

Spermatogenesis is an extraordinary complex process. The differentiation of spermatogonia into spermatozoa requires the participation of several cell types, hormones, paracrine factors, genes and epigenetic regulators. Recent researches in animals and humans have furthered our understanding of the male gamete differentiation, and led to clinical tools for the better management of male infertility. There is still much to be learned about this intricate process. In this review, the critical steps of human spermatogenesis are discussed together with its main affecting factors.

Keywords: spermatogenesis, sperm, reproduction, infertility, genetics, testis, testosterone, varicocele

1 Introduction

Spermatogenesis encompasses a complex network of processes that occur in the seminiferous tubules (STs) and culminates in the production of the mature male gamete. The processes are: proliferation of spermatogonia; spermatogonial differentiation into spermatocytes; meiotic division of spermatocytes producing spermatids; maturation of round spermatids; and the release of highly specialized mature spermatozoa into the testicular tubule lumen[1].

The entire spermatogenic process is thought to require approximately 74 days[2, 3], but a more recent study in normal men concluded that the total time to produce ejaculated sperm might vary between 42 to 76 days[4]. The estimated daily sperm production per man ranges from 150 to 275 million spermatozoa[3, 5].

Several testicular structures and cells play important roles during spermatogenesis, while an ample array of factors can influence its quality and quantity. In this article, we review the current literature regarding the main actors of human spermatogenesis.

2 Structures and cells

2.1 Seminiferous tubules

The average volume of an adult human testis is 30mL and its parenchyma is divided in 200 – 300 lobules by septations arising from the tunica albuginea. Each of these lobules contains one to three loops of STs, each one measuring 70-80cm in length when stretched, and with a total length of 250 meters per testis [1, 6]. The STs are the functional unit of the testis and occupy two-thirds of the organ's volume. They consist of a basement membrane, Sertoli cells (SCs), and germ cells at varying stages of maturation. So far, 13 types of human germ cells (GCs) have been identified. Classified in ascending order of maturation, they are the dark type A spermatogonia, the pale type A spermatogonia, the type B spermatogonia, the preleptotene, leptotene, zygotene, and pachytene primary spermatocytes, the secondary spermatocytes, and the Sa, Sb, Sc, Sd1, and Sd2 spermatids[7].

A peritubular tissue surrounds the ST and is composed of layers of myoid cells, fibrocyte-like adventitial cells, and collagen matrix. Together with SCs, this peritubular tissue forms the blood testis barrier (BTB)[8].

2.2 Peritubular myoid cells

Peritubular myoid cells (PTCs) are large, flat mesenchymal cells with some fibroblast-like and smooth muscle cell-like characteristics[8]. They are arranged in discontinuous cell layers, providing support for the STs. One of their main functions is the propulsion of the testicular fluid containing immotile spermatozoa towards the rete testis, using contractile filaments of actin and myosin[9, 10].

PTCs are also implicated in paracrine regulation of SCs functions. PModS (peritubular modifies Sertoli) is a PTC-derived molecule that modulates the secretion of transferrin, inhibin and androgen-binding protein by SCs. Other factors produced by PTCs that regulate SCs function are IGF-1, bFGF and a number of interleukins. PTCs also take part in the production and maintenance of the blood

testis barrier trough the secretion of fibronectin, collagens, proteoglycans and entactin[11, 12].

Several factors regulate PTCs activities. Studies using mice with the androgen receptor (AR) gene deleted in PTCs showed that androgens are important modulators of PTCs functions[13, 14]. In humans, AR are also found in PTCs, but to a lesser extent than in SCs. the clinical significance of androgen signaling in human PTCs is still unclear [15]. PTCs contractile function is regulated by angiotensin II acting via type 1 angiotensin receptor (ATR1) and oxytocin[10, 16, 17]. TNF-a strongly upregulates the production of proinflammatory interleukins by PTCs and might be related to the peritubular wall remodeling observed in some infertility patients[16, 18, 19].

2.3 Leydig Cells

Leydig cells (LCs) are located in clusters usually found in strategic positions between blood vessels and ST[20, 21]. They are polygonal in shape and exhibit ultrastructural features that are consistent with their function as the primary source of testosterone (T) in males.

LCs are thought to differentiate from fibroblast-like or mesenchymal cells in the testis interstitium[22]. In humans, their differentiation occurs in three waves, matching the also triphasic pattern of T production during development[23]. As a downstream event of sex-determining region (SRY) gene signaling, the first wave occurs between 8-18 weeks of gestation and is responsible for the male secondary sexual differentiation[24].

The second wave occurs in the first 2-3 months after birth in response to a concurrent LH surge. This wave is responsible for the hormonal imprinting of hypothalamus, liver, and prostate [1, 25]. The third wave is triggered by the maturation of the hypothalamic pituitary gonadal (HPG) axis during puberty. Originated from precursors cells and regressed neonatal LCs, adult LCs persist active throughout adulthood[23, 26].

Testosterone is the main LCs product, and the testes secret between 3-10 mg/day of T, accounting for more than 95% of total circulating T in post-pubertal men. Testosterone is synthesized from cholesterol in a complex process that involves multiple cytoplasmic and mitochondrial enzymes and transporters[27, 28]. The rate-determining step is the cholesterol transport from the outer mitochondrial membrane to the inner mitochondrial membrane by two particular molecules, TSPO (translocator protein), which acts as a channel[29], and the steroidogenic acute regulatory protein (StAR) whose mechanism of action is still unknown[30-32].

Testosterone produced by LCs may be stored in the intratesticular compartment or released into the systemic circulation. In humans, intratesticular testosterone (ITT) levels are 100-fold higher than serum levels[33, 34]. This may be due to local T production by Leydig cells combined with a countercurrent exchange mechanism in the pampiniform plexus[35]. Clinical studies on men using a T-based contraceptive regimen revealed that high ITT levels are necessary to support normal spermatogenesis[36] and that administration of hCG was able to bring ITT back to baseline levels in those men[37]. In addition, ITT optimization through the use of exogenous gonadotropins resulted in improved spermatogenesis in men with

non-obstructive azoospermia (NOA)[38]. There is no explanation for the need for such high levels of ITT in order to maintaining normal sperm production. In fact, much of the ITT might not be available to bind AR, since it is bound to androgen-binding protein, therefore, part of the ITT content might function just as a T reserve[39].

LCs are also the predominant testicular source of estrogens after puberty[40]. Estradiol is the main testicular estrogen and is metabolized from T by the microsomal P450 aromatase. This enzyme is responsible for maintaining the testosterone/estradiol ratio, and its activity is altered in some men with spermatogenesis failure[41]. Even though aromatase is mainly localized in LCs, it can be also found in SCs and GCs[42], the latter may be an estrogen source as important as LCs[43]. This widespread expression of aromatase highlights the importance of estrogens for spermatogenesis. Very high levels of intratesticular estrogens found in humans give even more support to this idea[44].

Other factors synthesized by LCs have been shown to influence human testicular function. Insulin-like factor 3 (INSL3) is a peptide produced almost exclusively by the LCs[45, 46]. Acting via the relaxin-family peptide receptor 2 (RXFP2), INSL3 mediates the migration of the testis to the scrotum[47, 48]. Its role in the adult human testis remains unknown, but a paracrine action on GCs is plausible. Importantly, INSL3 concentrations may reflect the functional status of LCS, possibly better than T concentrations[49, 50]. Oxytocin is also produced by human LCs and acts on the PTCs, increasing STs contractions. Oxytocin may also act in an autocrine way, stimulating steroidogenesis[17, 51].

The steroidogenic activity of LCs is driven by a series of hormonal and non-hormonal factors. Luteinizing hormone (LH) is the most important of them and acts by binding to high-affinity LH/HCG receptors on the plasma membrane. The binding of LH to LH/HCG receptors starts a chain of acute and trophic events. The acute events are mediated by coupled G protein - cAMP mechanism and include the synthesis of StAR and cytochrome P450 enzymes, leading to an immediate increase in the T and estradiol production within 24 - 72hrs[52, 53]. In contrast, the trophic effect is the inhibition of LCs apoptosis mediated through the ERK1/2 and Akt pathways[54, 55]. In addition to the hypothalamic/pituitary negative feedback, testosterone also regulates LCs function through an ultrashort loop negative feedback[56].

Other factors, such as gonadotropin-releasing hormone, melatonin, epidermal growth factor, insulin-like growth factor 1, atrial natriuretic peptide, ghrelin, and neurotransmitters, such as the gamma-aminobutyric acid, have also been reported to affect human LCs activity, but with less robust data[57-65].

2.4 Sertoli cells

Sertoli cells are considered the indispensable conductors of spermatogenesis and occupy 17–20% of the STs epithelium in an adult man[66]. They exhibit irregular shape and polarized alignment, with the base resting over the basal membrane and the apex pointing to the STs lumen[67, 68]. Another distinguished characteristic is the prominent nucleoli, which are readily identified in testis biopsy slides.

Their morphology is constantly changing, with vast cytoplasmic ramifications resembling tree branches, which involve GCs and assist with their migration towards the tubule lumen during spermatogenesis. A single SC can support up to 30–50 GCs at different stages of development[69, 70]. The framework for this intricate structure is provided by a complex cytoskeleton composed of actin filaments, tubulobulbar complexes, ectoplasmic specializations, intermediate filaments and microtubules, each one with its own ultrastructure and function.

Sertoli cells play a central role in the embryology of the testis. Until the sixth week of gestation, the human primordial gonad is fully bipotential, and the differentiation of SCs from progenitor cells of mesenchymal origin is the mark of male gonadal development[71, 72]. The presence of the Y-chromosome, or, more specifically, its SRY gene directs this unfinished organ towards testicular differentiation[73]. The product of SRY gene is a DNA-binding protein that upregulates a complex network of transcription factors, whose net effect is the differentiation of SCs. Other testicular cell types do not express SRY, and their male fate is directed by molecular signals arising from SCs[74, 75]. Shortly after differentiation, SCs start to produce anti-Müllerian hormone (AMH), which induces regression of the Müllerian ducts and precludes the development of female internal genitalia[76, 77]. SCs continue to secrete AMH throughout development and into adulthood, but the function of AMH after testicular differentiation remains unclear, with some authors suggesting a role in non-gonadal development[78, 79].

Androgen-binding protein (ABP) is another secretory product of SCs. It is a carrier protein that binds to T and dihydrotestosterone (DHT) with high affinity. ABP may regulate the bioavailability of androgens in the extracellular space of the testis and epididymis, maintaining a high concentration of these molecules in these organs. Others advocate that ABP also controls spermatogenesis and sperm maturation, protecting androgens from metabolism and facilitating androgen uptake in the male reproductive tract[80-83]. Transferrin, vitamin transporters, lactate, acetate, extracellular matrix components, glial cell-derived neurotrophic factor (GNDF), TGF-a, TGF-b and interleukins are other SC-derived products that seem to contribute to the microenvironment in which the GCs maturate[84-93].

Also produced by SCs, inhibin B is a glycoprotein hormone that regulates the production and secretion of FSH in the anterior pituitary gland, in a classic negative feedback loop[94]. Inhibin B is composed of two subunits, α and β. The α subunit is located predominantly in the SCs, while the β subunit is located mainly in the GCs, suggesting that an interaction between SCs and GCs is necessary for inhibin B production, at least after puberty[95-97]. Furthermore, inhibin B may be a paracrine and autocrine regulator of LCs and SCs functions[98-100].

Sertoli cell also act as macrophages, using phagocytosis to clean any degenerating GCs or residual bodies from spermatids. This is a critical function, because a considerable proportion of GCs are discarded during spermatogenesis, and the presence of these dead cells into the ST lumen could lead to the release of noxious contents that negatively impact sperm production[101-103].

Regulation of SCs activities is achieved by hormonal, non-hormonal and paracrine factors. The most important regulator of SCs is the follicle-stimulating hormone (FSH). Produced by the pituitary gland under the stimulus of

gonadotropin-releasing hormone (GnRH), FSH mediates the connection between the brain and SCs. Acting via specific G-coupled receptors, FSH activates several pathways, stimulating virtually all functions related to spermatogenesis. Despite the important role played by FSH, the hormone is not considered essential to spermatogenesis. Clinical data from men lacking FSH production or with FSH receptor mutation have revealed that spermatogenesis is heavily affected but not extinguished in these cases; a finding that could be explained in part by the discovery that the FSH receptor has some low level of constitutive activity[104-107].

In contrast, androgen signaling is indispensable to normal spermatogenesis, especially for meiotic progression and spermatid maturation, and SCs are the main mediator of androgen action in spermatogenesis[15]. The AR is found in SCs and moves to the nucleus to stimulate transcription when combined to its ligands (T or dihydrotestosterone). Unfortunately, the exact genetic mechanism by which androgens influence spermatogenesis is still unclear because few consistent target genes have been identified[108-111]. Therefore, secondary signaling pathways, such as Ca2+-dependent and MAP kinase pathways, have been proposed, but there is also paucity of data about them[105, 112, 113].

Insulin is another factor that modulates SCs activity, specifically, the carbohydrate metabolism (i.e. production of lactate). Insulin-deprived SCs were shown to have decreased lactate production, and, since lactate is the main energy source for some GCs, this effect could explain part of the deleterious influence of type I diabetes over spermatogenesis. Other substances, such as DHT, estradiol, melatonin, bFGF and interleukins, have been implicated in the regulation of SCs metabolism, but the their clinical significance is still unknown[87, 89, 114, 115]. As described earlier, PModS is a molecule secreted by PTCs that modulates the secretion of transferrin, inhibin B and androgen-binding protein by SCs. It has been shown to have more effect on SCs function than any other known regulatory factor, including FSH[12, 116].

2.5Germ Cells

Germ cells comprise a family of cells whose sole purpose is to become spermatozoa, and thus, to transmit genetic and epigenetic information across generations. They are the only human cell type capable of meiosis. Located within the STs, GCs are distributed in a highly organized manner, with less matured cells occupying the basal compartment and progressing to the adluminal compartment as they mature[3, 7]. The primordial GCs arise from extra-embryonic tissues surrounding the yolk sac. Between 3 and 5 weeks of development, they migrate to the gonadal ridge, where, directed by SCs factors, they differentiate into gonocytes.

The gonocytes enter in arrest in the G0 phase and stay mitotically inactive until after birth. Factors, such as the stem cell factor (SCF), stromal cell derived factor 1 (SDF-1), activator protein-2γ (AP-2γ), growth and differentiation factor 3 (GDF3), estradiol, retinoic acid, and methylation of DNA, appear to have an important role during the primordial GCs and gonocyte differentiations, and may be the source of some infertility causes and testicular tumors[117-127]. Between birth and 6 months, the gonocytes will further differentiate into spermatogonia (SPG),

which stay quiescent until the age of 5-7 years, when they increase in number via mitosis. Starting with puberty, and in parallel with the proliferation process, SPG begin the differentiation process towards spermatozoa[128, 129].

Spermatogonia are the diploid progenitors of all other GCs types, and have the dual responsibility of undergoing meiosis to produce the male gamete, and mitosis to self-renew, maintaining the continuous production of spermatozoa throughout man's life. Located in the basal compartment of the STs, and in close contact with SCs, they have an ovoid nucleus and a dense cytoplasm containing a small Golgi apparatus, few mitochondria, and many free ribosomes. SPG are divided in 3 subtypes based on their heterochromatin content: A dark; A pale; and B SPG[130, 131].

In the most accepted model, the stem GCs are the A dark and A pale SPG. The A dark SPG are the quiescent or reserve stem GCs, while the A pale SPG are the active stem GCs. The A pale SPG proliferate to either self-renew or to produce B SPG, which go through one mitotic division before initiating meiosis[132-134]. There is some debate about the pattern of A pale SPG division, but, using any of the current models, 8 preleptotene spermatocytes are produced from the original pair of A pale SPG[132, 135].

The decision whether the SPG will self-renew, differentiate or become apoptotic is mainly influenced by SC-derived factors. GNDF has been demonstrated to promote spermatogonial self-renew. In contrast, SCF, bone morphogenetic protein 4, retinoic acid and Notch1/Jagged2 signaling system induce differentiation. Several micro-RNAs are also implicated in the modulation of spermatogonial fate[93, 136-138]. Apoptosis is the mechanism used by SCs to control the number of GCs, keeping the proportion SCs/GCs constant. In fact, around 50-70% of developing GCs are discarded during spermatogenesis. The signal to GCs to enter into apoptosis is given by SCs trough the FaS/FaSL system, and modulated by Bcl-2, Bax and TNF-α[139-142]. The transcription factor nuclear factor κB (NF-kB) is likewise important in this process, but instead of acting directly on GCs, it induces transcription of SCs factors involved in the regulation of GCs death. Given their importance, any disturbance in theses mechanisms could affect the male fertility potential[143, 144].

Germ cells develop in consistent groupings called cellular associations. In humans, a system with 6 cellular associations, based on the morphological changes of the cellular nucleus after staining with hematoxylin-eosin, has been used since 1963[7]. Each cellular association is considered a stage in the spermatogenic cycle. Men with low sperm production might show atypical cellular associations, due to missing GCs or intermingling of associations[3, 5, 145]. Recently, a novel 12-stage cycle system was proposed using changes in shape and size of the acrosome made visible by immunohistochemical detection of the acrosin protein[146]. This system is similar to other ones described in non-primate mammals. In addition, there is some debate about the spatial organization of cellular associations. Animal models have shown that spermatogenesis is organized in waves throughout the STs. Early human studies failed to find the same pattern, and described human spermatogenesis as a chaotic process[3]. However, subsequent studies using computer modeling to map the distribution of primary spermatocytes proposed that

human spermatogenesis was organized in helical waves[147, 148]. Recently, 3D maps of cellular associations showed that their arrangement in humans was a random event[149].

Failure of GCs to develop further than the spermatogonial stage leads to azoospermia and a histological pattern called Sertoli-cell–only syndrome (SCO). SCO can be characterized by complete absence of SPG (SCO type 1), or by presence of rare foci of GCs with residual spermatogenesis (SCO type2). SCO type 1 is caused by disturbance of the migration of the primordial GCs from the yolk sac to the seminiferous cord, and exhibits intact basal membrane with numerous SCs in a very good morphological shape, but no GCs can be found. SCO type 2 is due to an insult occurring later on, allowing the persistence of some SPG, but also damaging other components of the STs. Despite the great reduction of the number of SPG and the presence of alterations in the other components of STs (i.e. SCs and basal membrane), sperm may still be retrieved using testicular microdissection techniques[150-152].

New promising technologies are been developed to help the identification of STs with undisturbed spermatogenesis in men with NOA. Multiphoton microscopy illuminates tissues with a near-infrared laser, which excites auto-fluorescence. This technique enables real-time imaging of tissue in-vivo without labels, providing high-resolution images that can be obtained at clinically useful depths with minimal damage to the tissue. The images can be used to differentiate between normal and abnormal spermatogenesis in human testis[153]. Other technique, Raman spectroscopy (RS), is a noninvasive and label-free optical technique that utilizes the molecular fingerprint of materials and transforms their biochemical information into a characteristic Raman spectrum. RS has been tested in animal and human models and has shown capability to distinguish seminiferous tubules with spermatogenesis from SCO tubules[154]. These technologies deserve further investigation into their clinical application to improve the sperm retrieval rates in patients with NOA.

Primary spermatocytes (PSCs) are produced by mitotic division of type B SPG and mark the end of the proliferative portion of spermatogenesis. They are the first GCs to cross the BTB to the adluminal compartment, where they become immunologically isolated and proceed to meiosis. PSCs are subclassified according to the stages of prophase 1 (i.e. preleptotene, leptotene, zygotene, and pachytene)[7].

The DNA recombination process occurs during the pachytene stage and is crucial to human evolution, therefore, quality control of meiosis 1 is very strict, with checkpoints during the pachytene and the transition metaphase1/anaphase1. Ten percent of men with NOA show a higher rate of recombination failure[155], while up to 45% of men whose partner suffer from recurrent pregnancy loss, and who have normal sperm density, motility, and morphology have increased sperm aneuploidy[156]. These findings could explain many cases of “idiopathic infertility”. Such infertile men might be at a higher risk of producing chromosomally abnormal offspring and should have genetic counseling[155, 157].

Another important player during meiotic recombination was recently identified. TEX11 is a germ cell-specific gene located in the X chromosome and encodes a protein that regulates homologous chromosome synapses and double-strand DNA break repair[158, 159]. TEX11 mutations were found in 2.4% of men with "idiopathic infertility" and were associated to spermatocyte apoptosis, maturation arrest and azoospermia[160].

After meiosis 1, two secondary spermatocytes are formed, each one with haploid number of chromosomes, but with 2n content of DNA. During meiosis 2, a single secondary spermatocyte is turned into two haploid/n round Sa spermatids[161, 162].

Maturation arrest (MA) during meiosis (early maturation arrest) is found in approximately 10% of the men with NOA. The histopathologic features of early MA include reduced tubule diameter and number of GCs, degenerating spermatocytes, and cellular debris[163]. Since meiosis 1 is more complex and has more quality control checkpoints than meiosis 2, MA at the stage of PSCs is more often found. Men with focal early MA may have some STs with normal spermatogenesis, and the overall surgical sperm retrieval rate in these cases ranges from 20-40% with microsurgical testicular sperm extraction (microTESE)[164-166].

As previously described, an important characteristic of spermatocytes and spermatids is their reliance on lactate as their principal energy source[87]. Glucose is taken up by SCs via the specific glucose transporter GLUT1 and then processed glycolytically into lactate, which is then transferred to GCs via monocarboxylate transporters. FSH, androgens, insulin and some paracrine factors stimulate this process, and disruptions can cause male infertility[167, 168].

Spermiogenesis is the maturation process by which one round Sa spermatid becomes one spermatozoon. During this stage, no more cellular divisions occurs, and a series of cytoplasmic and nuclear changes takes place. The acrosome is formed from the Golgi apparatus with participation of a cytoskeletal structure called perinuclear theca[169, 170]. Disturbances during acrosome development could lead to dramatic alteration in the head shape, called globozoospermia, a rare infertility condition that is difficult to manage. Globozoospermia is characterized by round-headed spermatozoa that are unable to penetrate and activate oocytes. Failure of inner nuclear proteins, mainly the DPY19L2 protein, to anchor the acrosome to the nucleus is the principal mechnism behind this pathology [171, 172]. DPY19L2 gene deletions are considered the most commom cause of globozoospermia, while SPATA16 and PICK1 mutations are also associated to this phenotype[173]. The outcomes of conventional intracytoplasmic sperm injection (ICSI) are poor in these men, since their sperm cannot active the oocyte. ICSI coupled with assited oocyte activation has resulted in live births[174].

The nucleus becomes more condensed and migrates to an eccentric position. Protamines replace 85% of the nuclear histone content and DNA is neatly packed around them in supercoiled structures named toroids, increasing DNA protection and becoming, in most part, transcriptionaly silent. In this setting of highly condensed DNA, protein synthesis relies on RNA stored in the cromatoid body. Some of these RNAs have been identified as non-coding RNAs, which have a role in

gene expression regulation. Poor DNA packing results in DNA fragmentation and decreases the fertility potential, impacting negatively on IVF outcomes[175-179].

The sperm tail arises from the centriole, gradually elongating the spermatid shape, and is formed of microtubules disposed in a 2 + 9 formation. In addition to the microtubules, other two structures, the outer dense fibers and the fibrous sheath, are organized and provide additional support and flagellar motion regulation. Mitochondria increase in number and gather around the tail basis, forming the midpiece [180-182]. In order to maximize spermatozoa motility, the excess of cytoplasm containing the remnants of Golgi apparatus and other organelles is removed, forming the residual body, which is phagocytized by SCs[183].

Spermiogenesis may be interrupted at any stage of spermatid development. When the arrest occurs at the early spermatid phase (dark round nuclei), the condition is called late maturation arrest, and when condensed, oval spermatids are present, it is called hypospermatogenesis (HS)[163]. Late MA seems to be less common than early MA, but carries a better chance of sperm retrieval with microTESE (50-80%), while hypospermatogenesis is found in up to 60% of men with NOA and has the best surgical sperm retrieval rate among all NOA histological patterns (80-90%)[163, 184, 185].

Once the spermatid is fully metamorphosed, it is detached from SCs and released into the tubule lumen in a process called spermiation. The details of spermiation in humans have yet to be elucidated, but it seems that the detachment of ectoplasmic specializations progress from tail to head, and that small tubulobulbar complexes maintain the last point of contact. This step is FSH and testosterone-dependent. Although defects in spermiation alone have not been found to cause human male infertility, they likely do contribute in some cases[104, 186, 187].

2.6 Blood Testis Barrier

Due to their high immunogenicity and specific metabolic demands, GCs need to be kept in a tightly regulated microenvironment. The BTB is an anatomical and functional barrier that divides the seminiferous epithelium in two separate spaces, the basal and adluminal compartments, and restricts the paracellular transit of substances[188, 189]. In mammalian testes, the most important components of the BTB are the cellular junctions between adjacent SCs, such as tight junctions, basal ectoplasmic specialization, gap junction, and desmosomes. Peritubular myoid cells and endothelial cells play a secondary role[190, 191].

The first cells to cross the BTB are the leptotene-PSCs, which migrate to the adluminal compartment to finish meiosis. Thus, meiosis initiation may not require the special microenvironment[192], but its consummation should occur in the adluminal compartment to avoid activation of the immune system by surface antigens of haploid GCs. Since GCs in the basal compartment also contain autoantigens, the BTB immunologic shielding seems to be reinforced by immunosuppressive activity of leukocytes and SCs[85, 193-195].

The transport of PSCs to the adluminal compartment, as well as the transport of round spermatids to the luminal edge, appears to be mediated by assembly and

disassembly of cell junctions in an orderly manner to prevent the passage of unwanted substances[85, 192, 196]. Furthermore, SCs transporters, such as efflux ATP pumps, mediate the flow of molecules, so that SCs are able to provide the nutrients needed by GCs, and to remove harmful substances. This mechanism is implicated in the resistance shown by some drugs to penetrate the BTB[197, 198].

Whereas several reports and animal models have suggested that the BTB is regulated by hormones, cytokines and growth factors, data gathered from the few human studies covering this topic are not enough to draw definitive conclusions. For example, animal studies have shown that intratesticular T acts as a trophic factor, however, in a human study, the suppression of intratesticular T did not disturb the BTB integrity. The study of BTB is an evolving field, but more human studies are needed. Advances in this area will likely bring us clinical tools applicable to humans in the area of contraception, male infertility and oncology[199-202].

3 Factors that directly affect human spermatogenesis

3.1 Obesity

Obesity has become one of the most studied health problems in the recent years. Using a body mass index (BMI) > 30 as the definition for obesity, the WHO estimates that 13% of the world's adult population was obese in 2014[203]. This scenario is even worse in the US, where a recent study showed a prevalence of 35% in adults[204]. Parallel to that, there is an ongoing debate about the possible relationship between the controversial worldwide decline in semen quality and the increasing number of obese men[205, 206].

Several studies have shown a negative association between sperm concentration/total sperm count and increasing BMI[207-210]. A negative impact on sperm morphology, motility, and DNA fragmentation has also been reported [211-215].

Several mechanisms have been proposed to explain how obesity could affect sperm production. The most studied mechanism is the hyperestrogenism. Levels of circulating estrogens are increased in obese men due to the increased peripheral conversion of testosterone to estrogens by the aromatase enzyme found in the adipose tissue[216, 217]. The excess of estrogens results in inhibition of LH and FSH by negative feedback on the hypothalamus and pituitary gland, decreasing T levels and the testosterone/estrogen ratio. With low T and FSH levels, spermatogenesis becomes defective. This mechanism is evidenced by the low levels of inhibin B in obese men[218-220]. Estrogen receptors (ER) have also been described in human testis. ERβ is found in almost all cell types of the interstitium and the STs, ERα is mainly found in LCs and SCs, and the G protein-coupled estrogen receptor (GPER) is present in PTCs, LCs and SCs. These findings imply a direct impact on testicular function[221-224].

Other mechanisms involved in the hypoandrogenism of obese patients include diminished levels of sex hormone-binding globulin (SHBG), insulin and leptin resistance, sleep apnea, and adiponectin deficiency[225-230].

Factors involved in the pathogenesis of obesity, such as high-calorie diets, sedentarism, genetic and epigenetic disorders, may also influence sperm production

[207, 231, 232]. Furthermore, some bariatric surgeries have been shown to impair semen parameters, probably due to malabsorption of vitamins and trace nutrients [233-235].

3.2 Diabetes

Characterized by hyperglycemia due to failure of insulin production and action, diabetes mellitus (DM) induces damage to various organs and systems, including the testis. Epidemiologic studies have shown that men with type 1 DM have significantly fewer offspring than their unaffected siblings[236], and that the estimated infertility prevalence in diabetic males ranges from 35% to 51%[237]. Despite the conflicting data about the impact of DM on classical sperm parameters[238, 239], there is some evidence pointing towards direct testicular damage. Diabetic men have increased DNA fragmentation[240, 241], which is probably due to oxidative stress originated from increased levels of advanced glycation end products, specifically Nε-carboxymethyl-lysine[242-245].

In addition, a recent study reported decreased lactate production by human SCs during insulin deprivation. This metabolic mechanism could directly affect spermatogenesis, since SCs-derived lactate has an anti-apoptotic effect and is the main energy source for spermatocytes and spermatids [114].

3.3 Environmental chemicals

That exposure to environmental chemicals (ECs) affects spermatogenesis has been suspected since the ancient times. Some Roman emperors and patricians had reproductive problems likely due to chronic exposure to lead from drinking contaminated wine[246, 247]. However, the definitive link between lead exposure and male infertility was not established until 1975[248].

Even though data from *in vitro* and animal studies corroborate the negative effects of ECs on the male reproductive tract, human epidemiological studies have been somewhat inconclusive.

There are many pathways by which ECs could disturb spermatogenesis, endocrine disruption is the most well known. Phthalates, chemicals used as plasticizers, have an anti-androgenic effect, decreasing the T production in LCs by activation of peroxisome proliferator-activated receptors (PPARs), as well as reduction of TPSO levels[249]. In addition, phthalates can also impair SCs and GCs functions[250]. Bisphenol A (BPA), a chemical used to manufacture polycarbonate plastics and epoxy resins, is another endocrine disruptor. BPA binds to AR, ERα and ERβ, exerting anti-androgenic and anti-estrogenic effects, and exposure to this EC has been associated to poor semen parameters[251, 252].

The BTB is likewise a target for ECs. Cadmium, a heavy metal used in the manufacture of batteries and pigments, has been shown to cause damage to the BTB, disrupting tight junctions and causing spermiation failure[253, 254]. Tetrachlorodibenzo-p-dioxin is a chlorinated hydrocarbon formed as a side product of herbicides and pesticides synthesis. Dioxin increases the c-Src activity, which alters the adhesive function of tight junctions and adherens junction in the BTB[255, 256]. Not surprisingly, men living in areas with high dioxin contamination have impaired semen parameters[257].

Alterations in the sperm chromatin structure is linked to some ECs, such as lead and ethylene dibromide. Lead is usually used as a pigment in paints and as a fuel component[258]. It binds to human protamines during spermiogenesis, altering sperm chromatin stability and potentially affecting normal chromatin condensation[259]. Ethylene dibromide (EDB) is a lead scavenger used in gasoline and was used as a fumigant. Studies with men chronically exposed to EDB showed a negative impact on sperm count, morphology and motility[260, 261]. Animal models suggests that EDB binds to histones, disturbing DNA packing[262].

Increased oxidative stress is also associated to some ECs. 1,2-Dibromo-3-chloropropane (DBCP) is a fumigant once used to control nematodes in field crops, but is still used in the synthesis of some fire retardants[263]. Men exposed to DBCP had decreased sperm count[264]. An in vitro study suggested that DBCP elevates ROS in GCs, inducing spermatogonial apoptosis[265].

3.4 Varicocele

Varicocele is an abnormal dilation of internal spermatic veins caused by incompetent venous valves, leading to reflux and stasis of venous blood. Varicocele can be found in 15% of all adult males, in 35% of infertile men and in 70-80% of men with secondary infertility[266]. Varicocele is considered a common etiology of male infertility, however, the cause-effect relationship between varicocele and infertility has not been conclusively established yet. Although studies confirm a negative impact of varicocele on fertility, studies of unselected men reveal conflicting results[267-270]. Despite the controversy, surgical treatment of infertile men with clinical varicoceles has been shown to improve semen parameters, pregnancy rates and testosterone levels[271-274].

Several hypotheses have been proposed to explain the potential negative effect of varicocele on spermatogenesis. Scrotal hyperthermia caused by venous blood stasis is thought to be the primary mechanism. Testicular functions are temperature-sensitive, therefore, intratesticular temperature should be kept 2 to 4° C lower than the rectal temperature[275]. Heat causes an increased rate of GCs apoptosis [276], likely mediated by decreased levels of cold-inducible RNA binding protein (Cirp)[277], as well as elevated levels of heat shock proteins[278]. High temperatures also impair testicular androgen production via a pathway involving increased oxidative stress damage to the LCs[279].

Recently, studies of the seminal proteome of men with varicoceles have highlighted the role of seminal components in the condition's pathophysiology. Adolescents with varicocele and abnormal semen parameters showed high expression of semenogelins I and II [280]. Semenogelins inhibit sperm motility and prevent premature sperm hyperactivation and capacitation, thus, they may be responsible, at last in part, for the decreased sperm motility found in these patients. The same group reported increased DNA-directed RNA polymerase III subunit (RPC2) levels in adolescents with varicoceles [281]. Since this protein is involved in the oxidative cascade, this reinforces the role of oxidative stress in the pathophysiology of varicocele. It is important to note that seminal proteome analysis has been extensively studied in infertile men with the objective of clarifying the importance of seminal components in several male infertility conditions, as well

as identifying biomarkers that could be used as diagnostic tools. For good reviews, refer to[282, 283].

Another potential mechanism for testicular function impairment is a decreased expression of E-cadherin and alpha-catenin proteins in SCs, with subsequent damage to the BTB and autoimmunity[284, 285]. Impaired disposal of residual sperm cytoplasm is also proposed as possible mechanism, resulting in defective sperm function [286, 287].

The histological findings in infertile men with varicocele include hypospermatogenesis, maturation arrest, decreased STs diameter, LCs hyperplasia, and testicular atrophy [288-290]. The effects on spermatogenesis seem to be bilateral, even in cases of unilateral varicocele, but the mechanism by which the contralateral testis is affected is unclear[291].

3.5 Genetic factors

Genetic disorders account for 15-30% of male infertility cases and might be responsible for the majority of "idiopathic" cases[292, 293]. Since the first reports of successful pregnancies after testicular sperm retrieval coupled with intracytoplasmic sperm injection of eggs (ICSI)[294], the interest on this field has significantly increased as specialists try to gather data that could be useful in the management and counseling of these couples. Notwithstanding the great advances in genetics over the last two decades, bench-to-bedside translation of this knowledge has been slow, hindering the development of diagnostic and therapeutic tools for clinical practice. This section will focus on genetic factors affecting human spermatogenesis with current, as well as potential future, clinical applicability.

3.5.1 Klinefelter Syndrome

Klinefelter syndrome (KS) is the most common genetic cause of male infertility, with a prevalence of 5% in men with severe oligozoospermia and 10% in men with NOA[295, 296]. Eighty percent of the men are 47,XXY, while the remaining have mosaic patterns such as 46,XY/47,XXY, 48XXXY or 48 XXYY[297]. The extra X chromosome has been shown to be of paternal origin in 60% of the cases. In this setting, X-Y non-disjunction during meiosis 1 is the most common error, and diminished X-Y recombination has also been described. For the maternal cases, non-disjunction due to errors in meiosis is associated with increased maternal age[298].

Despite the inactivation of an extra X chromosome in mammals, it is known that approximately 15% of the genes continue to be active in the silent chromosome. This process is skewed in patients with KS, resulting in an excessive genetic output that impairs androgen production and spermatogenesis[298, 299].

The main clinical characteristics of the KS are infertility, hypergonadotrophic hypogonadism, and cognitive disorders. The phenotype spectrum is wide, depending on the parental origin of the X chromosomes, the extent of genetic inactivation, and the presence of mosaicism[298, 300]. AR gene inactivation also seems to play an important role. Since the length of polymorphic stretch of CAG repeats contained in the exon 1 of the AR gene is inversely related to the receptor's activity, inactivation of the AR gene with a shorter or longer stretch of CAG repeats may be related to the severity of the syndrome[301]. Furthermore, a recent paper

reported that T production by LCs is normal or even increased in men with KS[302]. These evidences point toward a lower release of T into the bloodstream associated to the lack of responsiveness of the AR.

Regarding the impact of KS on spermatogenesis, a common feature is progressive degeneration of GCs and SCs, mainly after puberty[303, 304]. The mechanisms leading to testicular degeneration are still unknown, but overexpression of X chromosome genes, such as the angiotensin type-II receptor and the TEX11 genes, malfunction of FSH and androgen receptors, and increased aromatase activity may have a role[303, 305, 306].

Although men with KS and azoospermia show dramatic testicular alterations, the success rate of microTESE in finding viable sperm is 68%, and all the children born using IVF/ICSI with sperm from men with KS were healthy in one study[307]. This is probably due to small niches of undisturbed spermatogenesis composed by either a few GCs with normal karyotype, or by some 47,XXY GCs that are able to go through meiosis and produce sperm with normal karyotype (23,XY or 23,XX)[308, 309]. Despite that, the risk of using a hyperploid 24,XY sperm exists, and the conception of a 47,XXY fetus has been reported, thus, preimplantation genetic diagnosis is indicated[310].

3.5.2 Y-chromosome microdeletions

Located in the euchromatin zone of the long arm of the Y chromosome (Yq11), the AZF (azoospermia factor) region contains genes critical for spermatogenesis, among them, at least fourteen protein-encoding genes. These genes are divided in three groups based on their location: AZFa, AZFb and AZFc [311]. Microdeletions occurring in any of these zones have the potential to impair fertility and are found in 10% of men with non-obstructive azoospermia and 5% of those with severe oligozoospermia, but the incidence, and even the phenotypes, vary geographically and ethnically[312-314].

The AZFc group is located in the distal aspect of Yq11 and accounts for 60% of all Y-chromosome microdeletions (YCMD)[315, 316]. Several genes are located in the AZFc group, and the DAZ (deleted in azoospermia), a family of four genes implicated in spermatogenesis, has been the most studied[317]. The relative high incidence of *de novo* deletions via homologous recombination (HR) in this group is a product of the arrangement, similarity and the huge size of its amplicons, repetitive copies of nucleic acid sequences[318]. The most frequent deletion affecting the AZFc group is the one involving the amplicons b2 and b4 (b2/b4), which removes 8 genic families, including the DAZ family. Smaller partial deletions also exist and may happen either via HR, such as “b1/b3”, “b2/b3” and “gr/gr”, or via non-homologous recombination, such as P3a, P3b, P3c and P3b[316, 319].

Due to the wide variability of these deletions, the clinical and histological presentations are variable, but, in general, AZFc deletions are compatible with residual spermatogenesis. Patients may present with azoospermia or severe oligozoospermia, and histological findings vary from SCO, to MA and HS. Viable sperm can be found in up to 70% of the azoospermic patients that undergo microTESE[312]. Complete AZFc deletions could cause Y-chromosome loss and lead to 45X/46XY karyotype with Turner stigmata or sexual ambiguities. To avoid the

transfer of 45X0 embryos, preimplantation diagnosis should be offered to these couples[320].

Responsible for 15% of YCMD, the AZFb group is located from 18.1 to 24.7 Mb of the Y chromosome and contains the RBMY1 and PRY genes. The first is a testis-specific splicing factor expressed in the nucleus of spermatogonia, spermatocytes, and round spermatids, and the second is involved in the regulation of GCs apoptosis[316]. Complete deletions of this zone (proximalP5/P1) are massive, perhaps the largest in human genome. Homologous and non-homologous recombinations participate in these events, but other unknown factors may be also involved[321]. Since AZFb overlaps AZFc by 1.5Mb, combined AZFb + AZFc deletions occur and are, indeed, more frequent than isolated AZFb deletion[312, 322].

Patients with complete AZFb or AZFb + AZFc deletions show azoospermia, and testicular biopsy usually reveals SCO or diffuse early MA, hence, these patients must use donor sperm or adoption[312].

The AZFa group is located closer to the centromere and contains three genes: DBY, USP9Y and UTY. The DBY gene acts as a spermatogenic regulator during the earliest stages (i.e. spermatogonia), while the other two apparently are not crucial for male fertility[323, 324]. AZFa deletions have been shown to occur via intrachromosomal recombination between flanking repeats[325].

Complete deletion of AZFa is rare (3%) and carries the worse prognosis among all YCMD. Invariably all patients show azoospermia and SCO, and no sperm is found on microTESE. Therefore, these patients should not be submitted to invasive sperm retrieval procedures. In contrast, men with partial AZFa deletions often have HS and present with severe oligospermia or cryptozoospermia[312, 322]. We expect that genetic engineering advances will allow us to induce meiosis progression in stem cells, producing spermatozoa and helping men with complete AZFa, AZFb and AZFb+c deletions to father offspring in the future.

3.5.3 Micro RNAs

Micro-RNAs (miRNAs) are a class of short (20–23 nucleotides) single-stranded non-coding nucleotides, and constitute one of the most abundant ribonucleoprotein complexes in the cell. miRNAs exert a regulatory function over the expression of several protein-coding genes, and, therefore, modulate a wide array of biological processes. Their mechanisms of action are still under debate, but may include direct destruction of targets mRNAs, translation repression, and other indirect pathways to inhibit protein synthesis[326-328]. Due to the fact that the expression of miRNAs varies among different developmental stages, tissues and diseases, specific expression patterns could be linked to specific pathologies, and, thus, be used as diagnostic and therapeutic tools[329].

Regarding spermatogenesis, several animal studies showed that GCs miRNAs, many of them stored in the cromatoid body, are implicated in the regulation of apoptosis, proliferation and differentiation. This post-transcriptional regulation is essential because GCs are transcriptionally silent during certain stages of spermatogenesis. Alterations of miRNAs expression patterns could impair

spermatogenesis, and might explain a number of "idiopathic" male infertility cases[330-332].

Recently, efforts have been directed to associate specific miRNAs expression patterns in the seminal plasma with human testicular histopathologic patterns and clinical findings, with the idea of creating new diagnostic tools to assess the human male fertility[333]. So far 1,881 humans miRNAs have been described (www.mirbase.org)[334].

3.5.4 Epigenetic factors

Epigenetics is the study of several processes that alter gene expression without changing the DNA sequence. Some of these processes are DNA methylation, post-translational histone modifications, and chromatin remodeling. They can be cell, tissue, organ, sex and species-specific, can vary with different developmental stages, may be carried through generations and may be reversible[339].

DNA methylation occurs when a methyl radical is added to cytosine-guanine dinucleotides (CpG) by DNA methyltransferases. Areas of DNA with high content of CpG, called "CpG islands", have been found near promoters, and hypermethylation of CpG islands is associated with gene suppression, while hypomethylation is linked to gene expression[177, 340].

Histone methylation, acetylation, phosphorylation, ubiquitylation and sumotylation also modulate gene expression. Amino acid residues in the N-termini of histone tails are the sites for post-translational modifications, and several enzymes are involved in the process. The final effect, gene activation or suppression, depends on the combination among different sites and radicals[339, 341-343].

In addition, the manner by which DNA segments are packed around histones determines whether or not they are available for transcription. Tightly packed DNA segments found in heterochromatin are silent, while the loose DNA segments that constitute euchromatin are usually transcriptable, therefore, chromatin remodeling may activate or inhibit gene expression. The exact remodeling mechanisms are still unknown, but they appear to be regulated by ATP-dependent chromatin remodeling complexes[344, 345].

The DNA content in the male GCs is packed in a very small volume to fit into the sperm head. To accomplish that, 80% of the histone content should be replaced by protamines via hyperacetylation of histone H4 during spermatid stages. Highlighting the importance of this process, the degree of histone-protamine replacement has been correlated with the fertilizing capacity of the sperm, and decreased levels of H4 hyperacetylation were demonstrated in men with MA[346, 347]. Furthermore, the residual histone-bond DNA content is thought to be crucial for sperm function and early embryo development. The ratio between different types of protamine also affects fertility, as shown by studies demonstrating an increased DNA fragmentation in men with low P1/P2 ratio[339, 348-350].

A key event during spermatogenesis is the epigenetic reprogramming of GCs by widespread erasure of DNA methylation followed by *de novo* methylation. The reprogramming takes place during GCs differentiation during gonadal development, and during spermatogenesis, establishing a male germ line pattern of DNA hypomethylation. Not surprisingly, elevated DNA methylation at numerous

sequences has been associated with poor quality human sperm. However, the relationship between widespread DNA methylation and fertility is still a matter of debate[351-354].

Since some epigenetic alterations can be transmitted through generations, attention has been drawn to the association between assisted reproductive technologies and pathologies related to genomic imprinting, such as Prader-Willi, Beckwith-Wiedemann and Angelman syndromes. This may occur due to the use of defective sperm with incomplete reprogramming, or epigenetically imperfect oocytes arising from super-ovulation. Other cause may be ART procedures performed at the time of epigenetic reprogramming[177, 339, 350, 355].

Epigenetic mechanisms may also be the way by which several diseases and conditions, such as obesity and environmental exposure, affect spermatogenesis and influence the offspring[356-358]. However, further studies are needed to clarify these associations.

4 Conclusion

Advances in Andrology over the last three decades have paved the way for the elucidation of the molecular mechanisms that control human spermatogenesis. Rapid development of translational medicine and bench-to-beside collaborations are indispensable to further scientific knowledge. Much of the data reported here was first hypothesized in the clinical scenario, then elaborated using animal models, confirmed in human descriptive studies, and finally transported back to clinical practice via clinical trials. We still are far from completely comprehending the origin and function of the male gamete, but now we can have glimpses of this enormous universe.

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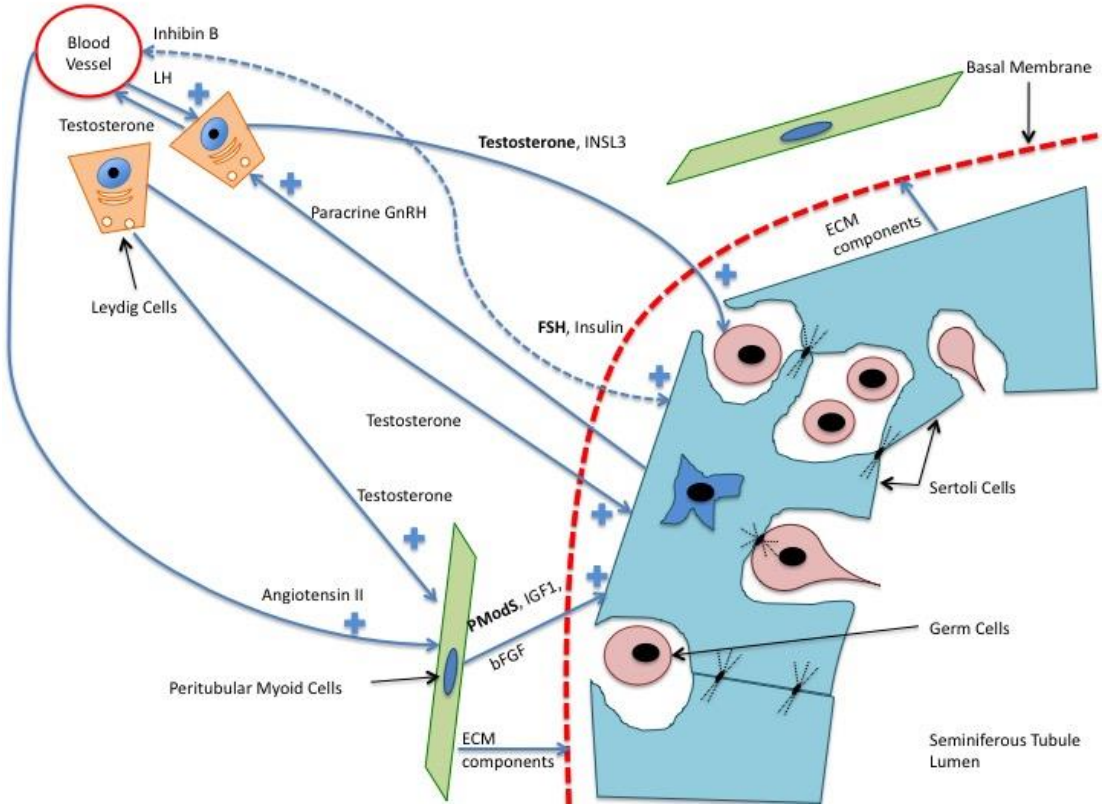

Figure 1: Hormonal and Paracrine Control of Spermatogenesis

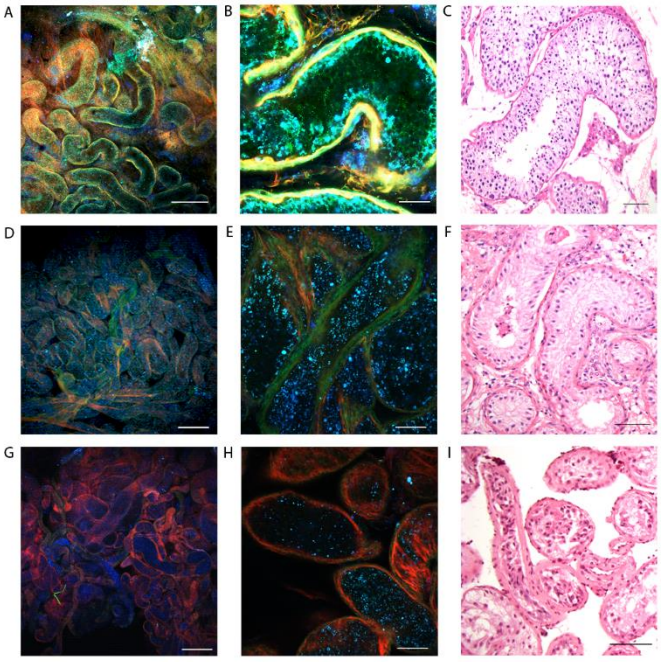

Figure 2. Different seminiferous tubular histology patterns imaged with multiphoton microscopy at lower magnification (A, D, G) and high magnification (B, E, H) compared to high magnification H&E stained tissue. Normal spermatogenesis(A, B, C). Seminiferous tubules with Sertoli cells only(D, E, F). Seminiferous tubules with Sertoli cells only and moderate peritubular fibrosis(G, H, I). Scale bar represents 500 microns for low magnification and 80 microns for high magnification. Reprinted from The Journal of urology 2012;Najari BB, Ramasamy R, Sterling J, Aggarwal A, Sheth S, Li PS, et al. Pilot study of the correlation of multiphoton tomography of ex vivo human testis with histology. 188:538-43 with permission from Elsevier

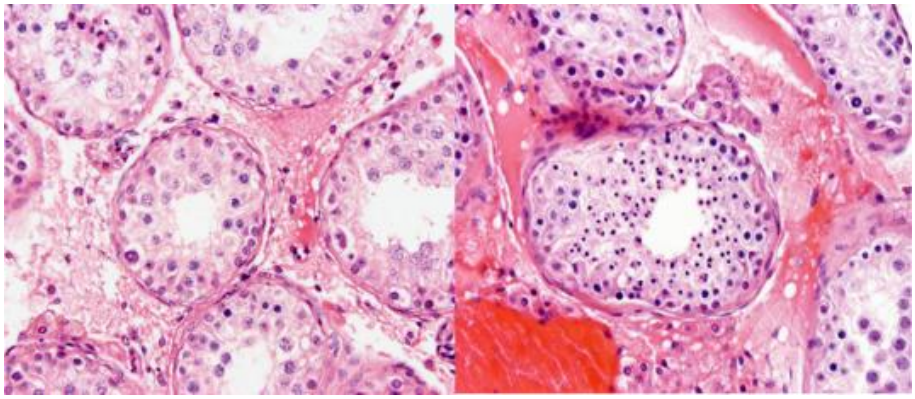

Figure 3: Early (left) and late maturation arrest (right). Reprinted from Fertility and sterility 2015. Bernie AM, Shah K, Halpern JA, Scovell J, Ramasamy R, Robinson B, et al. Outcomes of microdissection testicular sperm extraction in men with nonobstructive azoospermia due to maturation arrest. 104:569-73 with permission from Elsevier

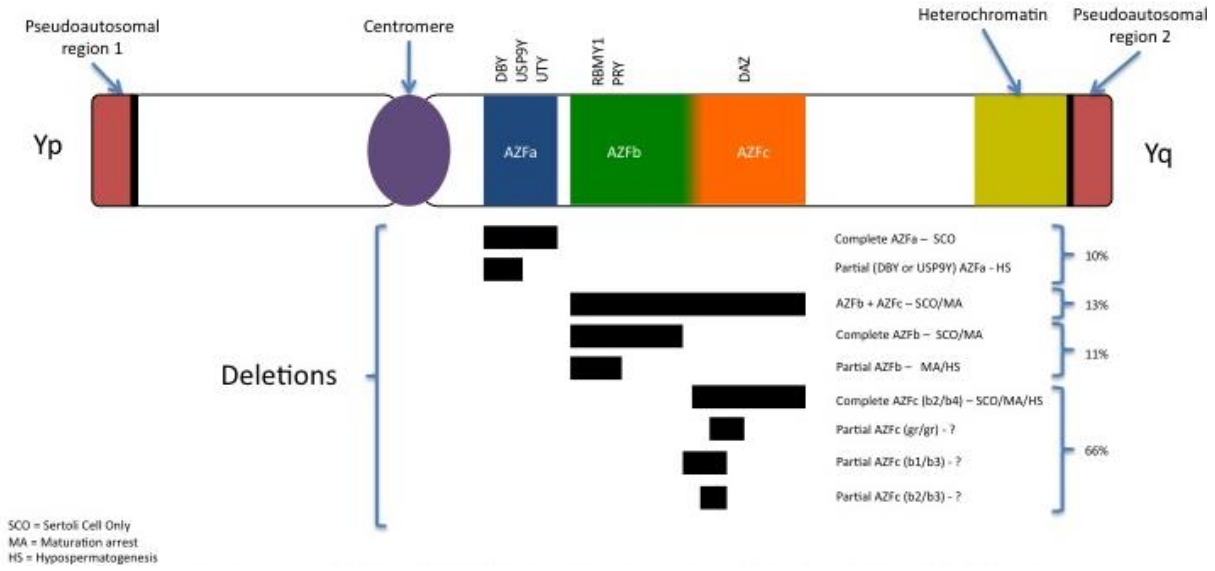

Figure 4: Y-chromosome microdeletions[293, 312, 314, 322].

Table1 summarize some of the ECs that affects human spermatogenesis:

Chemical Agent	Application	Exposure route	Effect
Phthalates	Plastics, tubings	Skin contact, inhalation	Anti-androgenic
Bisphenol A	Polycarbonate plastics and epoxy resins	Ingestion	Anti-androgenic, anti-estrogenic
Cadmiun	Batteries and pigments	Smoking, occupational	BTB disruption, Decreased T levels
Tetrachlorodibenzo-p-dioxin	Herbicides and pesticides	Occupational, ingestion	BTB disruption, Anti-androgenic
Lead	Pigment in paints and fuel	Inhalation	Chromatin disruption, damage to mannose receptors
Ethylene dibromide	Fuel component and fumigant	Inhalation	Chromatin disruption
1,2-Dibromo-3-chloropropane	Fumigant	Inhalation, skin contact	Increased oxidative stress

Table2 lists the most studied miRNAs in infertile men[329, 335-338].

Name	Function	Expression	Findings associated
miR-34 family	p53 tumor suppressor network	Down	NOA
miR-122	Suppresses the transcription of transition protein 2	Down	NOA
miR-19b	Inhibition of apoptosis	Up	NOA
Let-7a	Cell proliferation	Up	NOA
miR-181a	Regulation of T cell sensitivity	Down	NOA
miR-146b	Regulation of apoptosis	Down	NOA
miR-513a-5p	Regulation of apoptosis	Down	NOA
miR-509–5p	Regulation of apoptosis	Down	NOA
miR-374b	Oncogene	Down	NOA
miR-141	Regulation of cell cycle	Up	NOA
miR-429	Unclear	Up	NOA
miR-202-5p	Unclear	Down	NOA
miR-7-1-3p	Regulation of cell cycle	Up	NOA