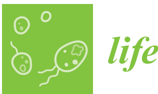 *life*

Review

The Fate of Leydig Cells in Men with Spermatogenic Failure

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Abstract: The steroidogenic cells in the testicle, Leydig cells, located in the interstitial compartment, play a vital role in male reproductive tract development, maintenance of proper spermatogenesis, and overall male reproductive function. Therefore, their dysfunction can lead to all sorts of testicular pathologies. Spermatogenesis failure, manifested as azoospermia, is often associated with defective Leydig cell activity. Spermatogenic failure is the most severe form of male infertility, caused by disorders of the testicular parenchyma or testicular hormone imbalance. This review covers current progress in knowledge on Leydig cells origin, structure, and function, and focuses on recent advances in understanding how Leydig cells contribute to the impairment of spermatogenesis.

Keywords: estradiol; Leydig cells; male infertility; non-obstructive azoospermia; spermatogenic failure; testicular dysgenesis syndrome; testosterone

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1. Introduction

The testicle is the primary organ responsible for maintaining male fertility and the hormonal state. Not only are these functions fundamental for the upkeep of male sexual characteristics, but they are also fundamental for fertility and species preservation [1]. The main cells engaged in maintaining these functions are Leydig cells (LCs), which reside in the testicular intertubular space, and Sertoli cells (SCs), which dwell inside seminiferous tubules [2]. SCs are responsible for supporting the regulation of the spermatogenesis process [3] and the supply of nutrients to germ cells [1]. LCs, on the other hand, are in charge of the synthesis and secretion of androgens, necessary for the masculinization of male fetuses, together with the initiation and maintenance of spermatogenesis [4,5]. In addition, LCs produce proteins with endogenous and xenotoxic metabolic functions, which also reduce oxidative stress, which thus protects the testicle from toxins [6,7].

Spermatogenic failure, manifested as azoospermia, is frequently linked to impaired LC activity [8–13]. Such deteriorated functioning is mainly manifested as histological and hormonal alterations in the testis compartment. These changes worsen with the severity of testis dysfunction [9,14]. Variations in androgen levels may be significant signs of either causal or developmental disturbances of both the androgen-producing and spermatogenic compartments of the human testicle [14]. Proper masculinization requires adequate testosterone (T) production early in fetal development, specifically in the masculinization programming window (MPW). In rats, experimentally-induced testicle disorders occur in response to disruption of fetal androgen production/action during MPW [15]. Subsequently, it has been proposed that there is a linkage between male reproductive disorders that occur at birth and those that emerge in adolescence [16]. To understand early susceptibilities to disturbance, we must first understand T production, including the cells involved.

The present review discusses recent insights from studies of the formation and function of LCs, as well as recent breakthroughs in understanding LCs contribution to diminished testicular function in spermatogenic failure.

2. The Development and Function of Leydig Cells

LCs are one of the most important cells in the interstitial tissue of the testicles. They play crucial roles in the development and function of the male reproductive organs [17]. The androgens produced by LCs are required for male genital differentiation and masculinization. Mammals, including humans, have two types of LCs, viz. fetal Leydig cells (FLCs) and adult Leydig cells (ALCs), which are present in the fetal and adult testes, respectively [18–21]. Some authors distinguish an additional neonatal Leydig cell (NLC) population in humans [21–24].

2.1. Fetal Leydig Cells

Although the origin of FLCs is unknown, several hypotheses have emerged. In rodents, FLCs are found in the testicular interstitium on gestational days 11–12 and are assumed to derive from numerous embryonic tissues, including the coelomic epithelium, migrating neural crest cells and mesonephric or epithelial populations of the neighboring mesonephros, early after testis determination. Evidence also suggests that FLCs and adrenal cortex cells share a common ancestor. However, none of the suggested progenitor pools have been shown to directly give rise to FLCs [18,25,26]. FLC differentiation is thought to be influenced by SC activity, and it has been demonstrated that SC products like desert hedgehog (DHH) or platelet derived growth factor (PDGFA) are essential for FLC development [26]. FLC numbers increase significantly during embryonic development, although these cells are not mitotically active. This means that new cells must arise as a result of progenitor cell differentiation and not the division of existing cells [25,27]. Human FLCs reach their peak differentiation at week 14, and maturation at week 18 of gestation. Later, up to the moment of full-term birth, FLC involution occurs [28]. So far, no information is available on the circulating factors that cause the degeneration of human FLCs. It is suspected that such signaling factors as anti-Müllerian hormone (AMH), gonadotropin-releasing hormone (GnRH), and transforming growth factor (TGF) may be responsible for the degeneration of FLCs in rodents [29,30]; however, their role in the regression of human FLCs is unknown [31].

FLCs are round to oval in form, and densely packed with lipid droplets. In the interstitium of prenatal rats, they tend to form clusters and are surrounded by a basement membrane containing collagen and laminin-based extracellular matrix. A similar discovery was made in humans, providing ultrastructural evidence for the basement membrane surrounding FLCs [32].

FLCs mainly produce androgen hormones that contribute to the development of male internal and external genitalia [18]. Testosterone (T), the most potent androgen in mammals, is derived from cholesterol through a chain of events mediated by a group of steroidogenic enzymes. Most of these enzymes are expressed in FLCs, except for one enzyme which mediates the final response to T production. Consequently, the major androgen produced by FLC is androstenedione, not T. Androstenedione is converted to T by fetal SCs [33,34]. In humans, between 8 and 20 weeks of pregnancy, T determines the formation of male internal genitalia from Wolffian ducts (also known as the mesonephric ducts) [35,36]. Human chorionic gonadotropin (hCG), secreted by the placenta, is the principal stimulator of T release until week 12 of pregnancy. Following this, luteinizing hormone (LH) receptors are expressed in FLCs, and after 16 weeks, T synthesis is controlled by LH [37]. The development of the external genitals occurs under the influence of dihydrotestosterone (DHT), which is primarily transformed locally from T by the enzyme 5-alpha-reductase [38]. The androgens produced by the FLCs and insulin-like factor 3 (INSL3) also influence the final stage in the development of the male gonad: the descent of the fully formed testicles into the scrotum [39]. The two phases of testicular descent are controlled differently, with the first transabdominal phase being largely dependent on INSL3, and the second inguinoscrotal phase being largely dependent on androgens. INSL3 acts on the relaxin family peptide 2 receptor (RXFP2), also known as leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8), in the gubernacular ligaments that connect the testes to the

inguinal region of the abdominal cavity [40–42]. INSL3, like T, is produced in a LH-dependent manner and is thought to be a sensitive marker of LC function and differentiation status [43,44]. INSL3 is strongly expressed in the fetal testis, but it is suppressed after birth, only to reappear after puberty [45]. This hormone is believed to have multiple functions in adult males since it operates as a paracrine mediator on reproductive cells as well as an endocrine factor elsewhere. Importantly, INSL3 levels appear to better indicate the functional condition of LCs than T levels [46].

2.2. Neonatal Leydig Cells

There is another generation of LCs in primate and human testes that form and vanish in a relatively brief amount of time during the neonatal stage. Although it is currently unknown where this generation of NLCs originate from, the existence of a triphasic pattern of human LCs development is now acknowledged as a working model [21–24]. The appearance of differentiated NLCs indicates the recruitment of precursors in the neonatal period. Some authors have suggested that the FLC population is involved in this phenomenon [28]. Moreover, the formation of NLCs and their function are thought to be governed by an increase in LH levels caused by hypothalamic–pituitary–gonadal (HPG) axis reactivation during the neonatal period. This is supported by the discovery of the presence of cells isolated from postnatal human testicles; these react more strongly to LH in the first few months of life than in the second and third year, with the simultaneous presence of FLCs [47]. The development of NLCs is associated with a significant increase in the production of androgens, mainly T. The exact physiological function of this short T-outburst by NLCs in humans is not known; however, it has been theorized that it may be important for imprinting different types of cells in androgen-dependent organs, e.g., the brain, to ensure their correct adult response to androgens [31].Over the next few months, the NLCs peak in number and, at the same time, the FLCs regress. Before the first signs of pubertal development appear, the HPG axis becomes inactive again and NLCs undergo involution or dedifferentiation [22]. The following resting phase in LC activity lasts for years until the development of the next, third generation of LCs, i.e., ALCs, begins [48].

2.3. Adult Leydig Cells

LC maturation is required for the initiation and maintenance of spermatogenesis, as well as for the promotion of secondary sexual characteristics in men [49]. ALCs arise after birth from a pool of stem cells known as stem Leydig cells (SLCs). The differentiation of LC from tissue stem cells to fully mature LCs with maximum steroidogenic capacity has been well characterized in rats. Adult rat LC differentiation is believed to occur in four consecutive stages; stem cells that differentiate into spindle-shaped precursor Leydig cells (PLCs), round immature Leydig cells (ILCs), and polygonal mature ALCs. These forms were discovered in studies on rats [50–52] and similar forms have been described in human testes [21]. The transition from SLCs to PLCs, ILCs, and ultimately ALCs is governed by LH, androgens, and numerous growth factors, including DHH, PDGFA, TGF, leukemia inhibitory factor (LIF), KIT ligand (KITLG), insulin-like growth factor 1 (IGF1), and fibroblast growth factor 2 (FGF2) [48]. ALCs, like other differentiated cells, have very little mitotic activity, and any recovery in the LC population depends on recruitment from tissue stem cells and/or LCs precursor pools [53]. SLCs can be found in the early interstitial compartment of the testicles shortly after birth. Additionally, spindle-shaped cells serving as precursors to ALCs have been found in the peritubular and perivascular regions of adult testes [54,55]. These cells were found to be able to self-renew indefinitely, or to specialize and eventually create T, just like the stem cells of the neonatal testis [20]. Based on a previous study on the induction of hypothyroidism in neonatal rats, it has been hypothesized that an increase in the number of LCs in the testes of such animals during adulthood occurs due to a simultaneous rise in the number of SCs [56]. However, in a study using a GnRH antagonist in neonatal rats, despite a significant decrease in SC numbers, no substantial reduction in LC nuclear volume or number per testis was observed in any

of the treatment groups in adulthood. This study also suggested that gonadotrophins, androgens, or estrogens did not significantly affect the LC number of treated rats during the first two weeks after birth. This could mean that the ultimate ALC number can be determined before birth [57].

2.4. The Relationship between Fetal and Adult Leydig Cells

The conjunction between FLCs and ALCs remains unclear. The first reports indicated that the postpartum FLCs niche completely regressed and was replaced by ALCs. However, this thesis has recently been disproved. The presence of FLCs can be found at the peak of hormone secretion, immediately after birth, in the so-called "mini-puberty" phase [58]. Then comes a period of quiescence for many years. During this period, most FLCs disappear or becomes morphologically unrecognizable [19]. However, some of them will survive and remain present in the mature testis [59,60]. Recent cell line-tracking studies in mice proved that FLCs can be found in the gonads of adult males, and they account for about 10% of the total LC population [61,62]. While these persistent FLCs appear grossly similar to developing ALCs, there are some subtle differences. FLCs have many large droplets of cytoplasmic lipids while ALCs have relatively smaller droplets in fewer numbers [18]. In addition, fetal and adult LCs exhibit different gene expression profiles. Correspondingly, it was hypothesized that LCs found in the fetal testis do not develop into adult cells. Instead, these two cell types arise as two separate lines with different functions and cell origins [48,63–65]. It is now believed that FLC and ALC progenitor cells are present in the fetal testes [65,66] and some researchers claim that FLCs and ALCs have the same set of precursors in the fetal testes [51,60]. While conclusive evidence is still lacking, recent research indicates the existence of a single ancestor that remains dormant until puberty and then forms ALCs. In addition, the origin of FLCs is currently not exactly known [67]. Final identification of the FLC source population is necessary to fully understand the genesis and nature of these cells. Currently, most of the evidence for potential FLC source populations is largely based on expression patterns of the characteristic factors that may define the lineage of the FLCs. The existence and function of FLCs, on the other hand, have recently been demonstrated to be significant in the creation and function of ALCs in a mouse model [68]. It is still uncertain if FLCs and ALCs are separate cell populations or share a common stem/progenitor cell lineage; nonetheless, stem ALCs have been found in the human fetal testis [19,69]. Despite this, the lineage interconnection between FLCs and ALCs remains uncertain, and the problem of the sequential evolution of two distinct LCs populations remains an issue [2,70]. Figure 1 presents a proposed model of LCs development and their function.

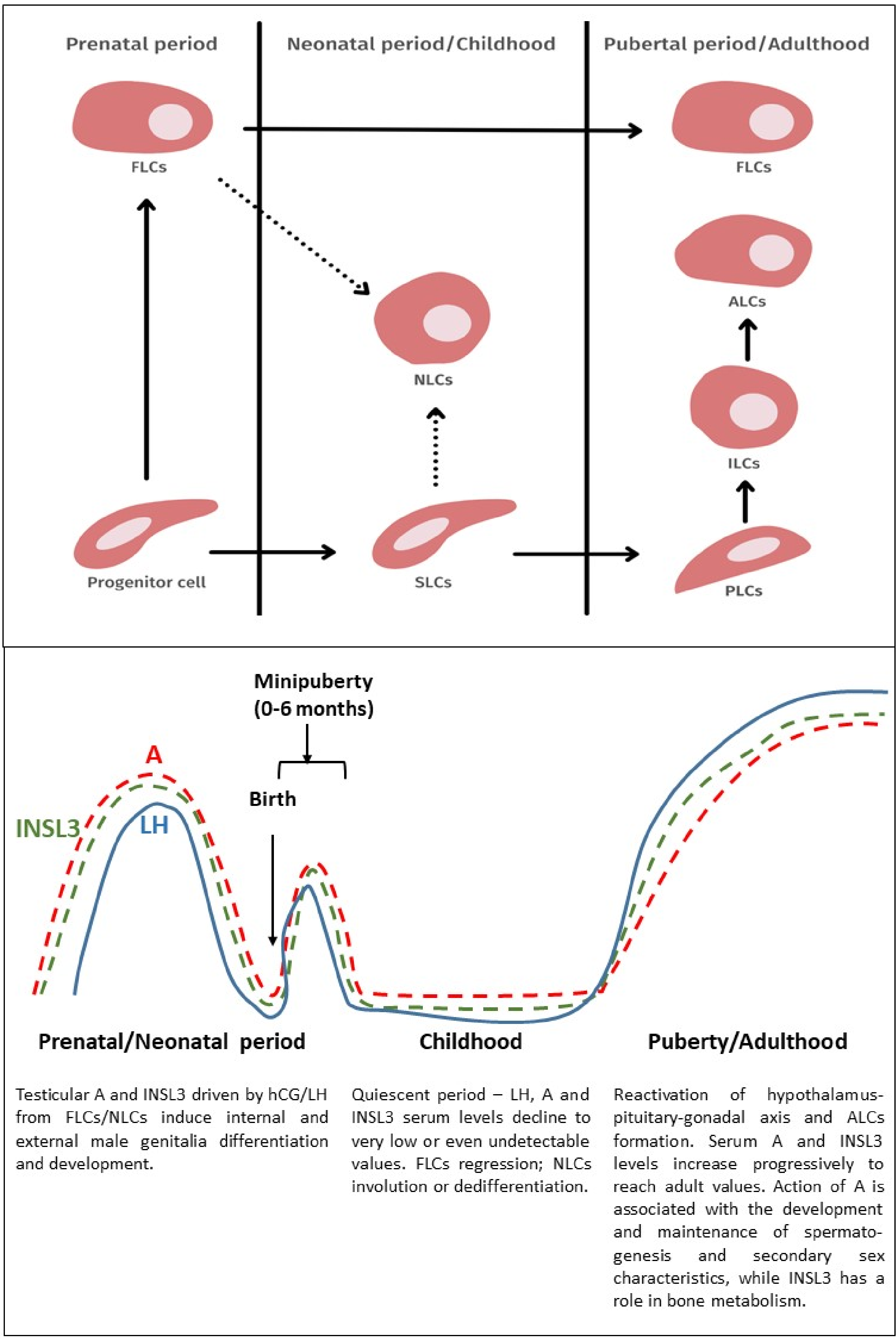

Figure 1. (**Upper panel**): Proposed model of Leydig cell lineage development. In the fetal testis, there is a shared Leydig cell progenitor pool that gives birth to both fetal Leydig cells (FLCs) and adult Leydig cells (ALCs). FLCs form in the fetal testis' interstitial area. In the neonatal testis progenitor cells

develop into stem Leydig cells (SLCs), which is the initial step in ALC subgroup differentiation. The dotted arrows show the hypothetical origin of neonatal Leydig cells (NLCs). NLCs are supposedly derived from either non-degraded FLCs or newly formed SLCs. NLCs slowly regress after the first postnatal year. During puberty, SLCs develop into mature cells via stages of newly progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and ALCs. A fraction of FLCs remains in the adult testis and accounts for approximately 10% of the total Leydig cell pool. However, it is still unknown how each progenitor is destined to become FLCs or ALCs. (**Lower panel**): Serum levels of luteinizing hormone (LH) secreted by pituitary and androgens (A) and insulin-like factor 3 (INSL3) produced by Leydig cells at different stages of their development. During the prenatal period fetal LH surges at mid-gestation, then declines and is low or undetectable in cord blood. The pattern of androgens and INSL3 concentrations follow that of LH. At birth, LH, androgens, and INSL3 levels are low and increase during the first weeks and months (minipuberty) to reach peak levels during the third month of life and then gradually decline and remain low at childhood. At puberty, LH, androgens, and INSL3 increase to reach the levels characteristic for adulthood. The main role of androgens and INSL3 in prenatal/neonatal period is induction of internal and external male genitalia differentiation and development. At puberty and then in adulthood, the main androgen action is associated with the development and maintenance of secondary sex characteristics and spermatogenesis, while INSL3 exerts a role in bone metabolism.

3. The Contribution of Leydig Cells to Spermatogenesis

Spermatogenesis is a complex process regulated by endocrine and testicular paracrine/autocrine factors. SCs play a major role in this process; however, new research has shown that other cell types play an equally important role in the development of reproductive cells [71]. Many studies indicate that many of the cells existing in the interstitial compartment of the human testes are involved in the regulation of the spermatogonial stem cells (SSCs) niche. These cell types are important for fetal testicular morphogenesis [72–74] and for spermatogenesis stimulation in adulthood to ensure male fertility [75]. The LCs drive spermatogenesis through the secretion of androgens, other hormones, cytokines, growth factors, transcription factors, and receptors associated with LCs [76]

3.1. Steroidogenesis

Steroidogenesis is the biosynthetic process of converting cholesterol into steroid hormones, including T [77]. Although they are synthesized mainly in the gonads, steroid hormones are also produced in the adrenal glands. In the adult testis, production of T in LCs is dependent on the pituitary gland's pulsatile release of LH into the peripheral circulation. In LC steroidogenesis, LH have two important roles: maintaining appropriate levels of steroidogenic enzymes and mobilizing and transporting cholesterol to the inner mitochondrial membrane. LH induces adenosine triphosphate (ATP) conversion to cyclic adenosine monophosphate (cAMP), which then catalyzes the production of protein kinase A, which is required for the transfer of cholesterol from the cytoplasmic pool to mitochondria. The peripheral benzodiazepine receptor (PBR) and the steroidogenic acute regulatory protein (StAR) transport cholesterol from the outside to the inner mitochondrial membrane [78,79]. On the matrix side of the inner mitochondrial membrane, cholesterol is converted to pregnenolone by the C27 cholesterol side-chain cleavage cytochrome P450 enzyme (CYP11A1). Then, in the smooth endoplasmic reticulum, pregnenolone is transformed to T via three steroidogenic enzymes: 3-hydroxysteroid dehydrogenase (3β-HSD), 17-hydroxylase/17,20-lyase P450 (CYP17A1), and 17-hydroxysteroid dehydrogenase type III (17β-HSD III) [77,80–82]. T synthesis from cholesterol can take place through several pathways. Normal adult testicular steroidogenesis in humans follows the Δ5 pathway, with a little amount of testosterone generated via the Δ4 pathway [83]. However, in rodents, testicular steroidogenesis primarily follows the Δ4 pathway [84]. Cholesterol in the Δ5 pathway is converted using the following intermediates: pregnenolone, 17-OH-pregnenolone, dehydroepiandrosterone (DHEA), and androstenediol. In contrast, the Δ4

pathway uses androstenedione as the last precursor to T. Androstenedione can be created by the conversion of DHEA as well as pregnenolone to progesterone and then to 17-OH-progesterone [85]. Small amounts of T or androstenedione are converted to estrogens in the testis by aromatase (CYP19A1). Figure 2 presents a schematic diagram of regulatory axis of testicular steroidogenesis.

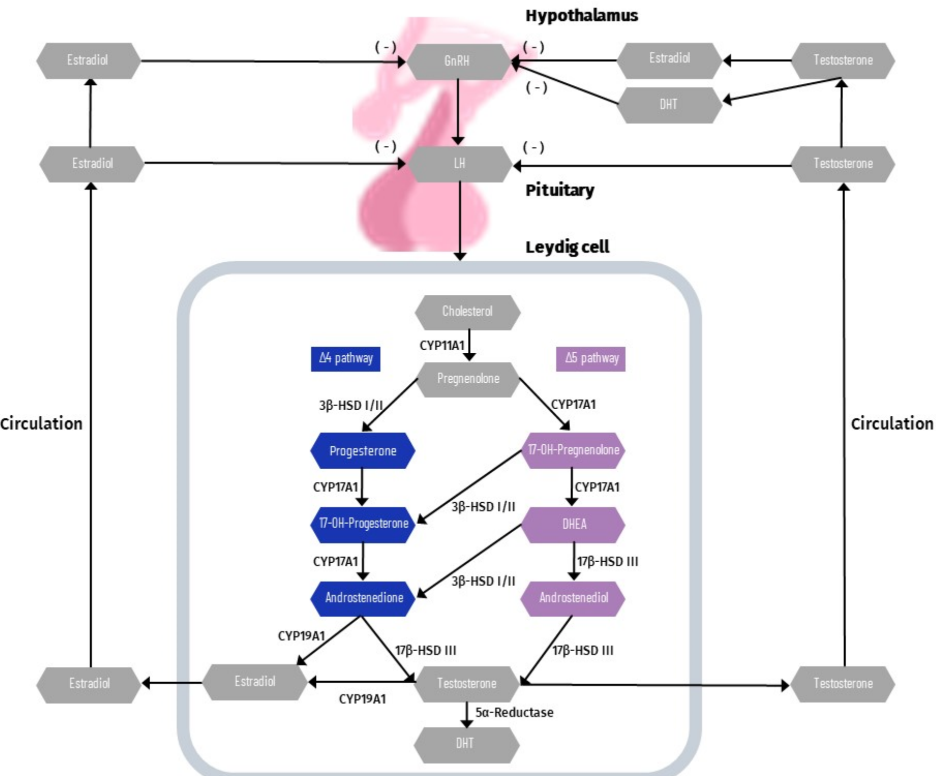

Figure 2. Schematic diagram of regulatory axis of testicular steroidogenesis. Sex steroids (predominantly testosterone) are produced upon central gonadotropin-releasing hormone (GnRH)—luteinizing hormone (LH) stimulation. LH binds to LH receptors on Leydig cells in the testicle and activates the pathway for the synthesis of steroid hormones from cholesterol. Different pathways variants are available after conversion to pregnenolone (Δ4 and Δ5). Normal, adult testicular steroidogenesis in men follows steroidal pathway Δ5, with a little amount of testosterone generated via the Δ4 pathway. Small amount of testosterone and androstendione are converted in the Leydig cells to estrogens by enzyme aromatase (CYP19A1) and to dihydrotestosterone (DHT) by 5α-reductase. Testosterone and estradiol act locally in testicle to regulate its function (e.g., spermatogenesis) or are released to blood circulation. Circulating sex steroids form a negative feedback loop to inhibit the secretion of GnRH and LH.

3.2. Testosterone

LCs are the major population of steroidogenic cells in the testicular interstitium and their undisputed contribution to male reproductive functions is the synthesis of T, which is fundamental for the progress of spermatogenesis [86]. This androgen, under the influence of the HPG axis, diffuses into the interstitial space and affects the signaling pathways of male reproductive cells by binding to androgen receptors (AR) [87–90]. It is known from mouse models that germ cells do not exhibit AR, despite the fact that they require androgens for survival and development [91]. Nevertheless, LCs and other somatic cells express AR, and it is generally assumed that these cell types mediate the testosterone needed for spermatogenesis [92]. T is required in at least four critical processes during spermatogenesis: blood–testis barrier (BTB) maintenance, meiosis, SCs sperm adhesion, and sperm release [93]. Analyses of AR-deficient SCs in rodent testes revealed the occurrence of three major fertility disorders: violation of BTB integrity, which exposes post-meiotic germ cells

to autoimmune and cytotoxic agents [94,95]; a blockage of the conversion of round sperm to elongated sperm due to a cell adhesion defect that causes the premature detachment of round spermatids from Sertoli cells [87,96–98]; retention and phagocytosis of mature spermatozoa by SCs [97]. Another study involving knockout of AR in mice indicated that T signaling is essential for several types of somatic cells to uphold spermatogenesis and preserve male fertility [99].

3.3. Estradiol

Estrogen has been recognized as another critical regulator of spermatogenesis in various animals, including humans [100]. Estrogen receptor 1 (ESR1, also known as ERα), estrogen receptor 2 (ESR2, also known as ERβ), along with G protein-coupled estrogen receptor (GPER), are abundant in the testes [101–103]. The SCs are thought to be the first location of estrogen production in the testis, switching to the LCs during neonatal development, when a gonadotropin-regulated aromatase is present. Estrogen acts on cells possessing estrogen receptors (ER), thus inducing spermatogenesis. Human LCs have low ESR1 and ESR2 levels but high GPER levels [101].

The most important estrogen produced in the human testis is estradiol (E2). E2 appears to cause a variety of changes in LCs, depending on the stage of their development. In the fetal and neonatal testes, estradiol acts by blocking the morphogenetic development of LCs from precursor cells [104]. There is further evidence that E2 inhibits LC regeneration in the testes of adult rats administered ethane dimethylsulfonate (EDS) [105]. Moreover, E2 affects T production in LCs. It appears to do so by blocking the activity of many steroidogenic enzymes involved in the synthesis of T [106]. In addition, E2 acts as a germ cell survival factor [107]. Research conducted on aromatase-knockout mice suggests that dietary phytoestrogens may partially prevent abnormal spermatogenesis by reducing germ cell loss [108]. Apart from the survival of reproductive cells, E2 regulates their proliferation and differentiation as well as apoptosis [107,109–112]. Low sperm count and poor sperm function may be caused by ER mutations [113]. ER inhibitors cause atrophic seminiferous tubules and impaired spermatogenesis in mice [114]. These results, together with those demonstrating progressive disruption of spermatogenesis in aromatase knockout mice [115], indicate the importance of estrogens for the normal function of the adult testis.

3.4. Other Factors

Numerous studies have shown that LCs regulate spermatogenesis, not only through the action of T, but also through the secretion of other factors such as growth factors, cytokines, and other hormones [31,59].

The key mitogen involved in the development of many critical for spermatogenesis cells is IGF1 [116,117]. IGF1 promotes proliferation in various types of testicular cells including LCs, SCs, differentiated spermatogonia, and SSCs. Analyses of individual RNA-Seq cells from mouse and human testes showed that IGF1 is mainly expressed by interstitial cells, including LCs. Meanwhile, IGF1r, which encodes the IGF1 receptor (IGF1R), has been detected primarily on spermatogonia among germ cells [118–120]. Moreover, studies on the loss of IGF1 signaling in LCs have shown that it has a very detrimental effect on male fertility. According to a detailed developmental analysis in mice with loss of Igf1r, FLC function was normal, but ALC maturation and steroidogenic activity were impaired due to an accumulation of PLCs. Igf1r-deficent mice were infertile, with decreased testicular weight and poor virilization. All of these defects, with the exception of testis size, were absent in SCs-specific knock-out animals, showing that they were caused by Igf1r deletion in LCs [121].

Hormone oxytocin is another substance produced by LCs that plays an important role in the control of spermatogenesis. This is because of the regulation of steroid production in LCs [122]. Mice need oxytocin to produce and release sperm. In studies in mice deficient in

oxytocin, the release time of sperm in the seminiferous tubule was significantly delayed, which supports this thesis [123].

The previously described hormone INSL3 is another significant factor in the development of the sperm cells. By administering INSL3 to mice with induced gonadotropin deficiency, it was revealed that it can prevent germ cell loss [124]. Moreover, a correlation has been noted between serum INSL3 levels and persistent spermatogenesis in studies of experimental male hormonal contraception [125]. Consistent with these findings, INSL3 is closely related to spermatogenesis.

During spermatogenesis and steroidogenesis, the cytokine network in the testis also boosts cell growth and differentiation, assisting in the coordination of multifactorial interactions [126]. Studies in rats have found that an important cytokine, macrophage migration inhibitory factor (MIF), is produced by Leydig cells [127]. MIF is secreted into the testicular interstitial fluid by Leydig cells and interacts directly with Sertoli and peritubular cells as a regulating factor for their signaling [127,128]. Another factor, TGFβ, affects LC steroidogenesis, testis development, and spermatogenesis. One of approximately 40 cytokines in the TGF family, TGFβ has been found in the LCs and other somatic cells, as well as the reproductive cells of pigs, rats, mice, hamsters, and humans [129–131]. Studies indicate that TGFβ1 and TGFβ3 are abundant in human LCs, as well as their receptors. TGFβ3 has been shown to interact with TGFβ-Receptor II [132] and TGFβ1 with TGFβ-Receptor I [133]. TGFβ1 was not found in the seminiferous tubules. TGFβ3 and TGFβ-Receptor I were found mostly in elongated spermatids, but TGFβ-Receptor II was found only in pachytene spermatocytes and was weak in spermatogonia, spermatids, and SCs. In the SCs, only TGFβ-Receptor II was found. Finding TGFβ isoforms and receptors in the LCs and germ cells of the adult human testis indicates that they have a role in the regulation of spermatogenesis [129]. In addition, due to the partial action of endogenous IL1, Leydig cells activate the production of large amounts of the immunoregulatory cytokine IL6. Both IL1 and IL6 have the ability to control the development of SCs and spermatogenic cells [126,134]. Higher plasma levels of IL6 have been detected in infertile/immunoinfertile men compared to fertile men [135]. The relevance of cytokines and their receptors in testicular cells, as well as their biological origin, is highlighted by these observations. As a result, in male infertility, the concentrations of these autocrine/paracrine factors should be taken into account.

4. Spermatogenic Failure and Leydig Cell Function

The World Health Organization estimates that about 9% of couples worldwide suffer from fertility problems, and the male factor is responsible for up to 50% of cases [136,137]. Testicular failure is defined by impairment of the endocrine (T production) and/or exocrine (sperm production) functioning of the testis. Problems during spermatogenesis manifest as decreased or absent spermatozoa production [138–140]. Spermatogenic failure, characterized by a complete lack of sperm production, is recognized as the most severe manifestation of male infertility in humans [141]. Global prognosis show that azoospermia, i.e., the complete absence of sperm in semen, affects up to 10% of men struggling with infertility [142,143]. Azoospermia caused by damage to spermatogenesis is classified as non-obstructive azoospermia (NOA) [144].

4.1. Non-Obstructive Azoospermia

The etiology of NOA is either lack of sufficient testis stimulation by gonadotrophins or internal testicular damage [145]. Direct causes include genetic abnormalities, congenital anomalies, hypogonadotrophic hypogonadism, post-infectious conditions, oncological treatment, testicular trauma, and the involvement of exogenous factors (Table 1) [137,146–148]. The majority of cases, however, are due to unknown causes, which is often referred to as idiopathic NOA [149]. Idiopathic NOA is believed to be caused by genetic defects which have not been fully discovered. Recent global trends attribute an increasingly important role to the contribution of NOA development to environmental factors. Endocrine disrupting chemicals (EDCs) are exogenous compounds that interfere with the stability

and control of the male endocrine system, as well as that of the offspring. By crossing the blood–fetal barrier, several EDCs can impair the development of the genitals, thus disrupting embryonic development. Cross-generational inheritance is also possible with some epigenetic processes [150].

Table 1. Direct causes underlying non-obstructive azoospermia.

Etiology	Example	References
Chromosomal	Klinefelter syndrome Y-chromosome microdeletions	[11,149,151] [152–155]
Genetic	Autosomal monogenic factors (TEX11, NR5A1, SYCP3, MEI1, and others)	[156–165]
Hormonal	Kallmann syndrome Acquired hypogonadotropic hypogonadism Hyperprolactinemia Androgen resistance	[166] [167–169] [170,171] [172,173]
Developmental/Structural	Cryptorchidism Varicocele	[174–176] [177–182]
Radiation and toxins	Radiotherapy Chemotherapy Drugs EDCs (phthalates, bisphenol A) Alcohol abuse	[183–185] [184,186,187] [188–192] [193–200] [201,202]
Infections	Mumps orchitis Others	[203,204] [205–207]
Testicular trauma	Torsion	[208,209]
Other exogenous factors	Heat	[210,211]
Idiopathic	-	[212–214]

4.2. Histological Pattern

Men with NOA have different degrees of spermatogenic deterioration [215]. The size of the testes indicates the degree of spermatogenesis; thus, small testes suggest spermatogenesis failure [139]. Some research indicates that the vast majority of NOA patients demonstrate significantly decreased testis volume [12,216]. Normal adult testicular size is determined mainly by germ and SCs, rather than by LCs. However, the LC pool and functioning, as measured by circulating T, are closely related to testicular volume in both pubertal boys [217] and adult males [218]. The histological pattern of the NOA testicles may present a variety of disorders, including hypospermatogenesis (HYPO), spermatogenic cell maturation arrest (MA) at the spermatid, spermatocyte, or spermatogonia level, and absent germ cells in seminiferous tubules (Sertoli cell-only syndrome (SCOS)) [219–222]. A combination of the aforementioned conditions can be seen in the histological sections of NOA testicular biopsies on occasion, which is termed as "mixed atrophy" (MIX) of seminiferous tubules [223] (Figure 3).

Furthermore, biopsy specimens from males with NOA, particularly those with a history of cryptorchidism and/or numerous foci of testicular microlithiasis, may indicate intratubular germ cell neoplasia in situ (GCNIS) [224,225]. Men with NOA have an increased chance of developing testicular cancer [226]. Hypospadias, cryptorchidism, spermatogenesis impairment, and testicular cancer may have origins in poor prenatal testicular development [227–229]. Assessment of the spermatogenesis status shows that NOA patients have a much lower Johnsen score (JS). Semithin sections data revealed a significant loss of diverse spermatogenic cells. There is a clear relationship between the thickness of the lamina propria and the quantity of spermatogenesis in the tubule. Furthermore, prior to spermatogenic failure, the thickness of the lamina propria may increase owing to increased extracellular matrix buildup [230,231]. Progesterone accumulation

was seen in the testicular seminiferous tubules' thicker lamina propria and was linked to spermatogenesis impairment. In the seminiferous tubules of patients with poor spermatogenesis, the progesterone-charged lamina propria is typically thicker. As a result, a change in the metabolic pathway in the human testes is expected, leading to reduced spermatogenesis [230,232]. A study conducted in patients with azoospermia revealed a thickened basement membrane and a reduced diameter of the seminiferous tubules in patients with SCOS. Due to increased deposition of collagen fibrils in the extracellular space, the intact spermatogenic process may be unable to produce sufficient quantities of laminin in the presence of damaged testicular tissue architecture, which might explain the significant decline in seminal laminin in SCOS [233]. In a retrospective study using archival testicular biopsies, AZFa-deleted specimens were found to have a combination of normal to thickened tunica propria, reduced tubular diameter, normal to increased intratubular space, hyperplastic LCs, and SCOS or spermatogenic arrest in a very small number of tubules [152]. Similarly, our group observed a thickened tubular membrane in the testicular tissue of SCOS patients [12].

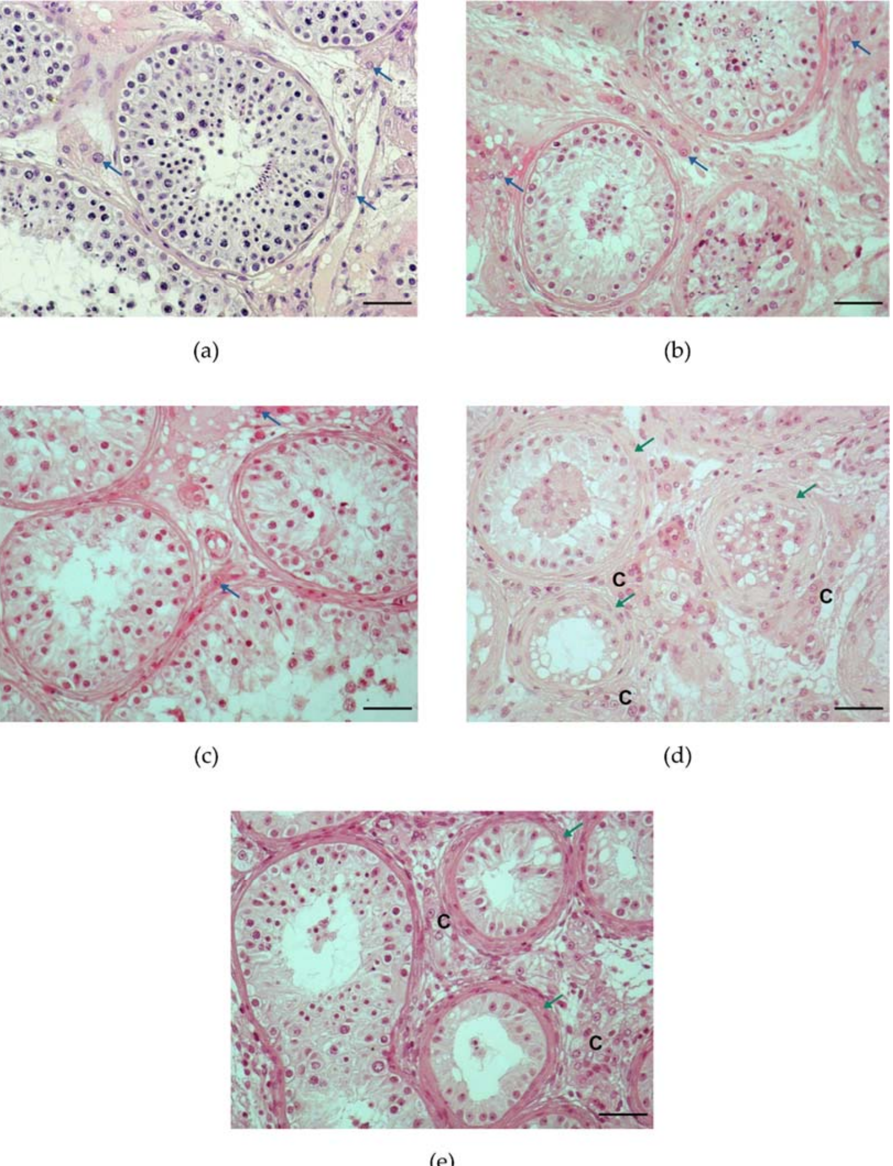

Figure 3. Microphotographs of testicular histology: (**a**) Section of testicular biopsy from men with obstructive azoospermia; normal human testis—well-developed seminiferous tubules with a clear

lumen, lined with seminiferous epithelium presenting complete spermatogenesis (qualitatively and quantitatively); a normal number of Leydig cells (LCs) is observed in the intertubular space; LCs are visible as solitary cells or are grouped in small clusters (2–4 cells) (blue arrow); (**b**) Section of testicular biopsy from men with non-obstructive azoospermia presenting hypospermatogenesis—in seminiferous tubules a general decrease in germ cell components is observed; a normal number of LCs is observed in the intertubular space (blue arrow); (**c**) Section of testicular biopsy from men with non-obstructive azoospermia presenting maturation arrest at spermatocyte stage—in seminiferous tubule germ cell development stops during meiosis, a normal number of LCs is observed in the intertubular space (blue arrow); (**d**) Section of testicular biopsy from men with non-obstructive azoospermia presenting Sertoli cell-only syndrome—significantly decreased tubule diameter, increased thickness of basement membrane (green arrow); seminiferous tubules are lined with mature SCs, no germ cells are present; in the intertubular space, an aggregation of LCs is present to form larger clusters (>5 cells) (C). (**e**) Section of testicular biopsy from men with non-obstructive azoospermia presenting mixed atrophy–seminiferous tubules presenting different histological pattern i.e., Sertoli cell-only pattern and hypospermatogenesis; in the intertubular space, an aggregation of LCs can be seen to form larger clusters (>5 cells); note the thickened basement membrane in tubules with Sertoli cell-only pattern (green arrow) in (**d**,**e**). Hematoxylin and eosin staining; Magnification—×200; scale bar, 50 μm.

NOA is usually accompanied by pathological changes in both the tubular and interstitial compartments. Although LCs and vessels make up the majority of interstitial tissues in normal testicles, those with spermatogenic failure have more interstitial tissue and excessive fibrous connective tissue [234]. Semithin sections of testicular biopsies from NOA patients exhibited notable vacuolization of some LCs, as well as macrophages stuffed with phagocytized material [216]. LCs may come into direct contact with testicular macrophages or lay near them. This demonstrates a complicated cellular interaction that may be one of the reasons for interstitial tissue changes in NOA [235]. The ultrastructure of LCs in patients with NOA changes significantly. Cells with the unaffected morphology can be detected in the same biopsies, although they are less in number. Because of the increased prevalence of heterochromatin in aberrant LCs, the nucleus was frequently indented. The cell's cytoplasm contains a large number of low electron density lipid droplets and vacuoles, as well as a decrease in the smooth endoplasmic reticulum cisternae, glycogen, and mitochondria [236]. Histological examination indicates Leydig's cells hyperplasia (LCH) characterized by a rise in the number of LCs with enlarged nucleoli and reduced lipofuscin. Hyperplastic LCs infiltrate between the seminal tubules and concentrate into multifocal nodules [237].

The changes in the LCs in NOA patients has been studied for many years. Some studies mention cell hyperplasia [13,195,238–242], while others excluded it [8,234,243–246]. Even though the proportion of testicular tissue occupied by LCs seems to be larger in histology samples from testes with dysgenetic features, when testis size is taken into account, there is often no significant increase in total Leydig cell volume [8]. Whether or not LCs are truly hyperplastic in men with NOA is still up for debate. In NOA men, LCs often form micronodules, which have been identified as a histological sign of spermatogenic failure. LC micronodules are defined as more than 15 LCs in a cluster [8,13,14]. This is a common finding in those with impaired spermatogenesis (Figure 4) [14]. The presence of large LC clusters has been linked to reproductive disorders such as Klinefelter syndrome [11]. In the testes of rats, phthalate-driven FLC aggregation caused by DBP was previously characterized as Leydig cell tumors [193,247]. Nevertheless, FLC aggregation is not a Leydig cell tumor since FLC quantity is unaffected by DBP exposure [248]. Findings in animals exposed to DBP in utero indicate that inappropriate aggregation of FLCs occurs most likely due to their irregular migration [248]. Another theory is that LC hyperstimulation occurs as a result of increased LH levels compensating for decreasing T levels, resulting in the creation of dysfunctional LC clusters, despite their large size [14]. Furthermore, histological heterogeneity was discovered in LCs coupled into micronodules, implying the presence of cells at various phases of development [53].

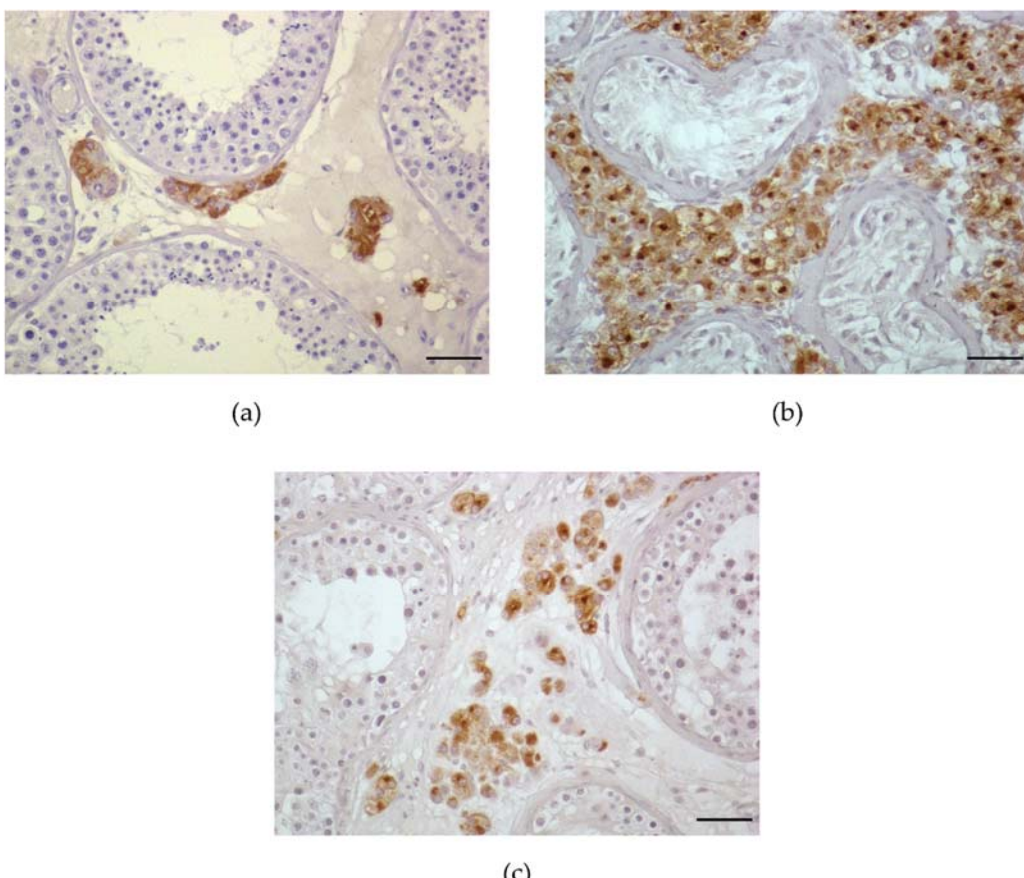

Figure 4. Immunohistochemical staining against insulin-like factor 3 in the mature Leydig cells in testicular biopsies from azoospermic men with different histological patterns. Insulin-like factor 3 expression at protein level is visible as a brown color in Leydig cell cytoplasm (used chromogen: diaminobenzidine—DAB); (**a**) complete spermatogenesis with normal Leydig cells number; (**b**) Sertoli cell only syndrome with exceedingly large Leydig cell hyperplasia; (**c**) mixed atrophy and increased Leydig cell number in the intertubular space. Magnification ×200; scale bar, 50 µm.

Gene set enrichment and protein expression analysis found that the maturation of SCs and LCs was impaired in patients with Klinefelter syndrome. These findings indicate that the cells are immature in adult males with Klinefelter syndrome, and that testicular function impairment begins early in development [249], as indicated by the discovery of the presence of spindle-shaped LCs in biopsies of men with NOA [234]. When compared to normal adult testis biopsy samples, the LC populations in testes with features of dysgenesis have a higher number of undifferentiated cells, as evidenced by increased delta-like homolog 1 (DLK1) and decreased INSL3 expression [53]. ILCs have fewer Reinke crystals (RCs) than mature ALCs, if any. This is consistent with animal studies that indicate immaturity in LCH [248,250].

4.3. Endocrine Profile

NOA is a substantial risk factor for androgen insufficiency [251–256]. In patients with NOA, serum T levels are commonly found to be low-normal or low [251,253,257]. Low T levels, decreased urogenital masculinization, higher risk of hypospadias and cryptorchidism, and reduced sperm production may all arise from insufficient androgen exposure during MPW [258,259]. The human testis generally generates very moderate amounts of Δ4-C21 steroids, as these are products of the Δ4 pathway: the Δ5 pathway is used throughout androgen production in healthy human testis. In testes with impaired spermatogenesis, however, the Δ4-C21 steroid progesterone builds up in the thickened lamina propria of the seminiferous tubules. Immunohistochemistry indicates that low JS testes express more

HSD than high JS testes. Yet, there was no difference in CYP17A1 expression levels across groups. Infertile testes display raised relative HSD levels in their LCs and adopt the Δ4 pathway to convert pregnenolone to T, instead of the Δ5 pathway. This metabolic pathway disruption might be caused by a change in LC density, since in the group with low JS, increased LCs density could enhance the expression of the HSD enzyme [260].

Low T levels are linked to high gonadotropin (LH, FSH) levels, leading to spermatogenesis problems [261–267]. Several researchers have linked spermatogenesis failure, such as SCOS, MIX, or testicular cancer, to LC dysfunction, which is marked by low T and elevated LH [8–10,13,268,269]. A deficiency of T in the body can interfere with spermatogenesis, resulting in spermatogenic cell loss and infertility. The number of SCs in adult men's testes is the most important factor of sperm production efficiency. The results of a study using mice lacking FSH, FSH receptors, or AR, reveal that both FSH and androgen are necessary for the formation of the full complement of SCs in adult males, while only androgens are essential during fetal and neonatal proliferation, and are required for specific transcript expression during prepubertal development [270]. Thus, reduced T release by FLCs might lead to the suppression of SC proliferation and, as a result, a decrease in the sperm output in adulthood. In some studies, a so-called compensated LC dysfunction was observed, characterized by elevated serum LH levels along with normal levels of total T [9,13,43,240,268,271,272].

It has been suggested that an increase in serum LH levels leads to LCH. Continuous LH stimulation of the LCs can lead to the destruction of the LCs. This overstimulation can result in the production of huge vacuoles and extensive cisterns of the smooth endoplasmic reticulum in LCs. Overstimulated LCs, as well as those with preserved normal form and function, produce enough T to maintain normal blood levels. Furthermore, overstimulated LCs may produce an excess of T, which might explain why a small number of NOA patients had elevated T levels and T overexpression as determined by immunohistochemistry [216]. Since serum T levels may differ between NOA patients, a better indicator of LC function is T/LH ratio. Lardone and colleagues studied factors of LC function (T/LH ratio, Leydig cells/cluster size) along with measures of spermatogenic damage (FSH and testicular volume) in various histological patterns among patients [13]. The T/LH ratio was discovered to be substantially correlated with the number of LCs in a cluster, FSH levels, and testicular volume. These correlations were found to be comparable with the pattern of spermatogenic lesions. In addition, the number of LCs was positively associated with FSH levels and negatively related to testicular volume [13]. Hormonal imbalance has also been connected to the development of LCH and the severity of spermatogenic damage.

In tissues with severe spermatogenesis failure, smaller testicular volume and larger LCs clusters were observed [273]. This can lead to high testicular steroid concentrations per unit volume, but not enough to compensate for serum T levels, as compared to those with normal testicular volumes. As a result, each LC specific hormonal action appears to be altered [13]. In line with prior studies in cancer patients, it was found that the appearance of an expanded LC compartment and LCH in the biopsy contralateral to the tumor-bearing testicle was associated with long-term biochemical signs of LC dysfunction, reflected by decreased serum total T levels and lower total T/LH ratio [274].

Men with NOA have been found to demonstrate high-normal or high levels of E2, as well as a low T to E2 ratio, indicating enhanced aromatase activity [9,273,275,276]. Aromatase P450 (CYP19A1), which is encoded by CYP19, is responsible for the aromatization of androgens into estrogens in various tissues, including the gonads, brain, and adipose tissue. In human testicles, CYP19A1 was localized in LCs, SCs, and germ cells [273]. However, it is the LCs that are the primary site where T is converted to E2 during male adulthood.

Testicular CYP19A1 appears to be normal in the majority of men with poor spermatogenesis. However, immunohistochemical labeling revealed that men with NOA have higher levels of CYP19A1 transcript and protein expression, and that this was dependent on the severity of spermatogenic dysfunction. No relationship between serum T to E2 ratio or intratesticular testosterone (ITT) to intratesticular estradiol (ITE2) ratio was found,

although a link was found between lower ITT and increased ITE2, CYP19A1 expression, and sperm retrieval [277]. The link between CYP19A1 expression in LCs and the ITT/ITE2, but not the serum T/E2, indicates that local aromatase expression altered the intratesticular hormonal milieu [277]. In a certain subset of men with complete SCOS, an altered ITT/ITE2 ratio indicated an increased quantity of CYP19A1, which could lead to LC failure [273]. Additionally, in animal studies, increased ITE2 levels have been associated to LCH and/or LCs hypertrophy [238,278]. This was also confirmed in humans in a study where SCOS patients with low T/LH ratio and LCH had overexpression of CYP19A1 in LCs, which resulted in higher ITE2 levels [279]. Figure 5 presents a schematic illustration of estrogen synthesis in testes.

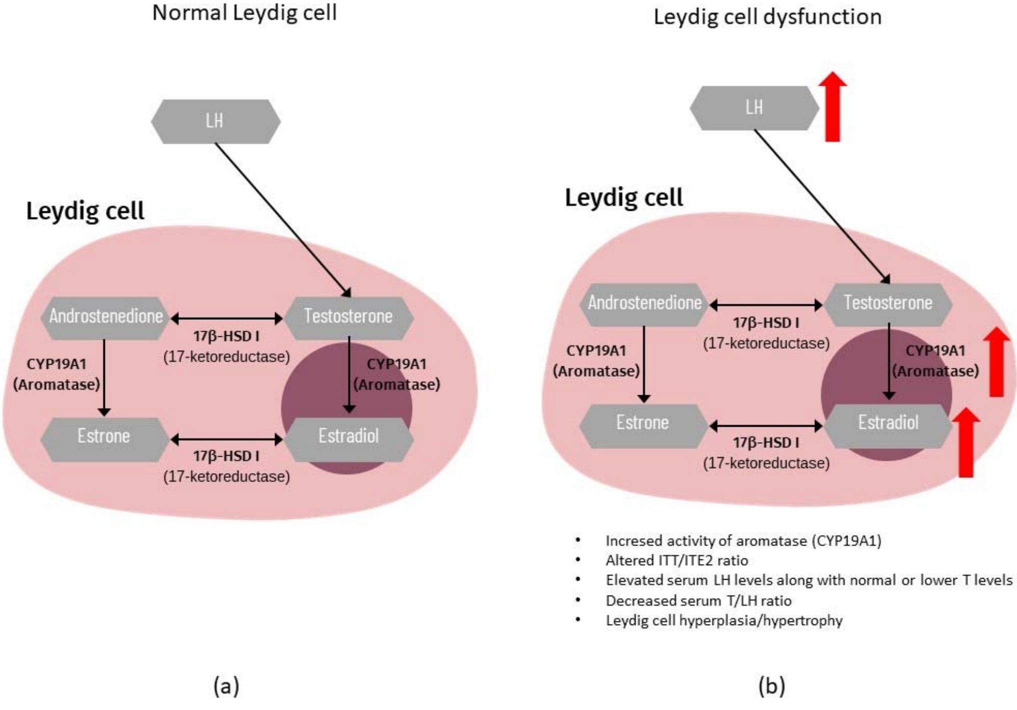

Figure 5. Estrogen synthesis in testes. (**a**) In adulthood, the main site of testicular estrogen production is the Leydig cell. Under the LH stimulation, testosterone (T) and androstendione are converted by aromatase (CYP19A1) to estrone and estradiol (E2), respectively. The proper balance between intratesticular T level (ITT) and estradiol level (ITE2) is crucial for normal testicular function (steroidogenesis and spermatogenesis); (**b**) In testes with impaired spermatogenesis, the dysfunction of Leydig cells is observed. Leydig cell dysfunction is accompanied by increased serum LH levels and decreased T/LH ratio. It was reported that in dysfunctional Leydig cells, the increased activity of aromatase may be observed, which results in elevation of intratesticular E2 levels and thus altered ITT/ITE2 ratio. The increased ITE2 level may be associated with Leydig cell hyperplasia/hypertrophy.

A recent study examining the quantity of estrogen sulfotransferase and steroid sulfatase mRNA in testicular tissue from SCOS patients found that an unbalanced steroid sulfatase/estrogen sulfotransferase pathway contributes to the testicular hyperestrogenic milieu in individuals with spermatogenic failure and LC dysfunction [280]. Moreover, decreased androgen production in SCOS was associated with lower CYP17A1 expression, which is affected by E2. These findings show that elevated E2 levels in some SCOS patients may play a role in the post-transcriptional degradation of steroidogenic activity in LCs.

Finally, LCs from men with SCOS and symptoms of testicular steroidogenic dysfunction demonstrated a mismatch in the transcriptional and protein expression of CYP17A1, which is associated with lower T synthesis and increased ITE2 [281]. Apart from the clear effects of CYP17A1 mutations and total CYP17A1 deficiency in men with missing or partial external genitalia development [77], milder variants of testicular CYP17A1 deficiency have

been associated to idiopathic infertility and hypospadias [282]. Disturbances in male reproductive tract development are caused by a disruption in the androgen–estrogen balance rather than by the action of estrogens alone [283]. Major developmental abnormalities of the male reproductive system related to exposure to potent estrogens can only occur when androgen activity is inhibited at the same time. The latter is brought on by T deficiency [57], LC development complications [57] and, most importantly, the decrease of AR expression [284,285]. It has also been revealed that the progesterone receptor (PR) and ER have a role in the pathophysiology of the MA and SCOS phenotypes in infertile males. In MA and SCOS patients, PR expression was decreased in all cell types when compared to OA patients, with only the truncated variant of PR present in SCOS. ER expression was decreased in the spermatogenic cells and LCs of MA testes, but enhanced in the LCs of SCOS testes [286]. Mizuno et al. [287] conducted a study on cryptorchid rats with impaired spermatogenesis. They hypothesized that higher ESR1expression in the LCs of cryptorchid testes is related to E2 levels in the testicular tissue, and that an androgen–estrogen imbalance impairs spermatogenesis in cryptorchidism.

Along with T and E2, INSL3 is a crucial secretory product of testicular LCs. Serum INSL3 reflects LC maturity and function [288]. Inhibiting the HPG axis alters INSL3 production [44]. INSL3 levels in the blood can be reduced in people with hypogonadism, such as those with Klinefelter syndrome [43,289,290] and Kallmann syndrome [291]. Mutations in the INSL3 or RXFP2 genes cause improper testicular descent during embryogenesis in rats, and it is a rare cause of cryptorchidism in humans [42,292–294]. INSL3 circulating concentration has mostly been evaluated as a possible marker for LC activity and as a potential prognostic tool for developmental and reproductive disorders in men [295]. T and E2 may influence INSL3 gene transcription by attaching to their receptors, activating or inhibiting transcription factors SF1 and NUR77, respectively [295,296]. The INSL3 promoter contains a testosterone-responsive element that acts as a binding site for NUR77 and SF1 [297]; several hypotheses about the role of estrogens have been proposed, including estradiol-mediated disruption of NUR77 and SF1 acetylation status and antagonism between AR and ER, but the exact mechanisms remain unknown [298].

4.4. Testicular Dysgenesis Syndrome

Growing epidemiological evidence points to a direct cause-and-effect relationship between prenatal estrogen exposure and various pathologies of the male reproductive tract, such as hypospadias, cryptorchidism, abnormal spermatogenesis, and testicular cancer, which have been grouped together under the name *testicular dysgenesis syndrome* (TDS) [16]. Testicular dysgenesis during fetal development can result in a primary failure of spermatogenesis and impairment of LCs. Micronodules are a common finding in those with impaired spermatogenesis and other TDS-related disorders [15]. In normally descending TDS testes, LCs within micronodules demonstrate a lack of RCs, which may be a characteristic of recently renewed ILCs [299]. Altered serum levels of reproductive hormones and reduced size and volume of male reproductive organs are other characteristics, which may also be part of the syndrome [300,301]. Although TDS and NOA share many common characteristics, not every case of TDS will be associated with simultaneous occurrence of NOA and vice versa. Men with TDS may only have slightly reduced semen quality and be fertile or subfertile [300]. According to the TDS theory, genetic and/or environmental factors cause a failure in SC and LC differentiation as a fundamental change in early fetal development. As a result, germ cell proliferation and T production are impeded [16,302]. TDS is also associated with altered estrogen levels. Cryptorchidism, epididymal abnormalities, infertility, and testicular cancer have all been linked to estrogen and xenoestrogen exposure during fetal and neonatal development [303]. A growing body of epidemiological evidence suggests a direct causal link between prenatal estrogen exposure and a variety of male reproductive system disorders grouped together as TDS [16]. Moreover, TDS is also associated with a mutation of INSL3 [45].

5. Conclusions

In summary, infertile men with spermatogenic failure exhibit substantial evidence of LC dysfunction. The association between impaired spermatogenesis and altered LCs activity might be related to an aberrant paracrine connection between the seminiferous epithelium and LCs. On the other hand, impairment of spermatogenesis and LC function may result from congenital insufficiency of both compartments arising during abnormal fetal/infant development. LCs micronodules are prevalent in human testicular biopsies, specifically in regions with severe spermatogenic insufficiency and an imbalance in the hormonal ratios, promoting the paracrine connection hypothesis between germ cells and LCs. Animal models were designed to investigate the mechanisms underlying diseases associated with prenatal-initiated spermatogenesis failure, as these events are difficult to analyze in humans. These trials provided compelling evidence of a link between somatic cell dysfunction and diseases associated with NOA and/or TDS. However, the mechanism by which defects in fetal testicular development cause reproductive difficulties in adult males, such as infertility, still remains unknown. Much research has been done on the origins and development of the LCs since they were originally reported in 1850 by Franz Leydig. Still, little is known about the ontogenesis of LCs and the relationship between FLCs and ALCs. Determining how LCs are formed will undoubtedly help to understand the development of the male reproductive system, as well as the mechanisms underlying TDS and T deficiency, and how environmental factors can affect the male reproductive system.

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