REVIEW

Gonadotoropin actions on spermatogenesis and hormonal therapies for spermatogenic disorders

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Abstract. Microdissection testicular sperm extraction and intracytoplasmic sperm injection have made it possible for men with non-obstructive azoospermia (NOA) to conceive a child. A majority of men cannot produce sperm because spermatogenesis *per se* is believed to be "irreversibly" disturbed. For these men, it has been thought that any hormonal therapy will be ineffective. Further understandings of endocrinological regulation of spermatogenesis are needed and LH or FSH receptor knock out (KO) mice have revealed the roles of gonadotropin separately. Spermatogenesis has been shown to shift during evolution from FSH to LH dominance because LH receptor KO causes infertility while FSH receptor KO does not. High concentrations of intratesticular testosterone secreted from Leydig cells, ranging from 100- to 1,000-fold higher than in the systemic circulation, has pivotal roles during spermatogenesis. This is especially important during spermiogenesis, a post-meiotic step for progression from round to elongating spermatids. Sertoli cells are the target of FSH and have numerous androgen receptors, indicating that Sertoli cells are regulated by FSH and the paracrine functions of testosterone. In combination with Leydig cell-derived growth factors, particularly epidermal growth factor-like growth factors, Sertoli cells support spermatogenesis, especially at proximal levels of spermatogenesis (*e.g.*, spermatogonial proliferation). Taken together, the current knowledge from human studies indicating that testosterone optimization by clomiphene, hCG and/or aromatase inhibitors and high dose hCG/FSH treatment can, at least in part, improve spermatogenesis in NOA. Accordingly hormonal therapy may open a therapeutic window for sperm production in selected patients.

Key words: Non-obstructive azoospermia, Testicular sperm extraction, Gonadotropin, Hormonal therapy

FIFTEEN to twenty percent of couples are categorized as infertile. Generally, a male-related factor is involved in more than half of the cases. Among male-related factors, approximately 10 % of cases are diagnosed as non-obstructive azoospermia (NOA). NOA is the most prevalent and challenging form of azoospermia for both patients and physicians because spermatogenesis *per se* is believed to be "irreversibly" disturbed and it has been thought that any medical therapy, including hormonal therapy, will be ineffective. Unlike oogenesis, where there is a substantial amount of information regarding endocrine regulation, information regarding spermatogenesis, especially in humans, is very limited.

Along with the development and availability of intracytoplasmic sperm injection (ICSI) since the

1990's, the goal of NOA treatment has not necessarily required many sperm in the ejaculate for assisted insemination or in-vitro fertilization. However, a single sperm is theoretically enough for ICSI to perform infertility treatment. As a result, the established surgical approach to retrieve sperm directly from the testis was called microdissection testicular sperm extraction (micro-TESE) [1]. The procedure is the best way among several modified sperm retrieval methods [2]. Generally, the sperm retrieval rate (SRR) performed by an experienced surgeon is 40–50 %. However, we cannot provide any treatment for the patients for whom the procedure could not retrieve sperm because their testicular histopathology is mainly classified either as maturation arrest (MA) and/or Sertoli cell-only (SCO). The pathophysiology of NOA is heterogeneous and some, but not all, patients respond to hormonal therapy including anti-estrogens, aromatase inhibitors and gonadotropins. Such immature germ cells can potentially be further developed as represented by late MA or hypospermatogenesis histology [3].

Spermatogenesis is regulated by a complex array of endocrine, paracrine and juxtacrine regulatory cross-talk that involves Sertoli, Leydig, peritubular and germ cells. Gonadotropins, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), play pivotal roles by stimulating Leydig and Sertoli cells, respectively. Ethane dimethane sulfonate (EDS), GnRH-deficient (hpg) mice and hypophysectomized animals have been used to study the roles of gonadotropins. However, the precise mechanisms relating to LH or FSH individually have been demonstrated by using gonadotropin knock out (KO) and receptor KO mice. To establish a novel gonadotropin therapy to stimulate impaired spermatogenesis, understanding the roles of each gonadotropin on spermatogenesis in normal and pathological conditions is crucial. In this review, we summarize the roles of Leydig and Sertoli cells and the mechanisms of gonadotropin-driven regulation of spermatogenesis mainly obtained from *in vitro* studies and gene-modified animals. In this review, available human clinical data regarding normal human spermatogenesis and spermatogenic disorders are linked to information from these basic studies.

Role of LH in spermatogenesis

LH stimulation of Leydig cells

LH has a pivotal role on testosterone production by stimulating Leydig cells, which exist outside seminiferous tubules and possesses LH receptors (LHR). The main role of Leydig cells is testosterone production. Recently, transcriptome analysis of testicular tissue of LH receptor KO (LuRKO) and testosterone-treated LuRKO mice showed that most of the defects in gene expression in the testis in the absence of LH actions were largely corrected by testosterone administration [4]. This indicates that testosterone production is the main role of Leydig cells. Adequate spermatogenesis is not achieved by testosterone administration. The rapid effects of LH on Leydig cells to produce testosterone last for less only a few minutes. Testosterone is produced from progesterone from a cholesterol precursor followed by an increase in intratesticular cAMP concentrations. The rate-determining step is performed by steroidogenic acute regulatory protein (StAR) which are present in the mitochondrial outer membrane. Chronic stimulation of LH is also required to maintain gene expression of StAR [5]. *In vivo*, gonadotropin is secreted rhythmically under the regulation of GnRH and the central nervous system. However, the baseline is not zero, indicating that activation and expression of StAR are regulated by the combination of acute and chronic stimulation of LH. It is easy to understand that testosterone has a paracrine role within testis for the maintenance of spermatogenesis. On one hand, testosterone is secreted into the systemic circulation and undergoes sexual (libido, erectile and ejaculatory) and anabolic (muscle volume, bone density, *etc.*) functions. The primary target of testosterone, the androgen receptor (AR), exists throughout the whole body, mainly in the male genitalia. In the testis, Sertoli cells are the target cells for testosterone and dihydrotestosterone (DHT) through the AR [6]. Sertoli cells secrete testosterone-dependent paracrine stimuli for germ cells. Sertoli cells are also the target of FSH through FSH receptors (FSHR), indicating that Sertoli cells are regulated by FSH stimulation and paracrine effects of testosterone to support spermatogenesis.

Testosterone is one of the most important factors for initiating and maintaining spermatogenesis [7]; however, the precise mechanisms supporting spermatogenesis are poorly understood. With LHbeta KO, the phenotype was that of a normal male. However, the mice were infertile with retarded growth of male accessory reproductive organs such as the epididymis and prostate and spermatogenesis halted at the round spermatid stage [8]. It was long believed that FSH was more important for spermatogenesis than LH. However, findings form LuRKO mice revealed the importance of LH action for spermatogenesis. Accordingly, the main regulatory gonadotropin for spermatogenesis has been shifted from FSH dominance to LH dominance [9].

Role of testosterone in spermatogenesis

The main testosterone-dependent steps during spermatogenesis are called spermiogenesis, the post-meiotic step for progression from round to elongating spermatids [7, 10-12] in the final phase of spermatogenesis. Spermiogenesis is highly dependent on intratesticular testosterone (ITT) in rodents [10]. Similar to the results with LuRKO mice, Sertoli cell-specific AR (SCAR) KO mice presented with infertility and maturation arrest during the meiotic process [13, 14]. This animal model histologically resembles MA in men with NOA and supports the observation that SCAR expression is lower in humans with early MA compared to late MA [15].

Additionally, high ITT is necessary for transition from type A to type B spermatogonia, an early step during spermatogenesis [7]. Testosterone generally seems to have a minor role in spermatogonial proliferation [16, 17] but is involved in the survival of spermatocytes and spermatids, presumably through anti-apoptotic mechanisms [18]. On the other hand, Hazra *et al.* developed transgenic gain-of-function Sertoli cell-specific AR mice and observed premature postnatal spermatogenic development with increased levels of post-meiotic germ cells [19]. These findings are very important in the clinical setting because we can increase ITT by using hCG, clomiphene citrate or aromatase inhibitors. Actually, azoospermia treatment that obtained mature sperm by administering only hCG has been reported [20]. Additionally, pre-operative optimization of ITT could increase SRR by micro-TESE [21]. In humans and rodents, AR have been reported to localize to the nuclei of Sertoli cells, peritubular myoid cells, Leydig cells and fibroblasts [22-25]. Several studies have described AR localization in germ cells, mainly in spermatogonia and spermatocytes [26, 27]. Using human testicular biopsy specimens, we confirmed the absence of AR immuno-expression in germ cells as well as intense staining in Sertoli cells [15].

The classical testosterone action on Sertoli cells involves DHT binding with AR and subsequent direct regulation of targeted genes by the AR-DHT complex. Using rat Sertoli cell primary culture, Fix *et al.* reported that testosterone rapidly activated MAP-kinase pathway and CREB transcription factors in an AR-dependent manner, indicating that testosterone can also act on Sertoli cells in a non-classical manner [28]. Recently this group found that testosterone-bound AR rapidly phosphorylates SRC following ERK phosphorylation and then modulates the expression of CREB target genes [29]. This non-classical testosterone action was provisionally proposed as important for spermatogenesis because spermatogenesis is blocked during meiosis *in vivo* if this non-classical pathway is specifically suppressed even if the classical testosterone signaling pathway is active [30].

Suitable ITT concentration is an enigmatic issue in many animals and humans. ITT is present in high concentrations within the testes, at concentrations ranging from 100 to 1,000-fold higher than found in systemic circulation, as reported by several laboratories

[31]. On the other hand, Pakarainen *et al.* reported that 5-10 % of normal ITT is enough to initiate and maintain spermatogenesis [32]. Using LuRKO mice, Oduwole *et al.* reported that exogenous testosterone administration with 5 mg but not 2.5 mg increased ITT level up to 20 nmol/L, which is still lower than control (120 nmol/L) and could restored sperm production [33]. In other words, the testosterone level in testes has a variety of effective ranges, and spermatogenesis is thought to be executed within a wide range with a varied extent of sperm production. ITT is reported as 150 times higher than that of systemic circulation in humans [31] and 25 to 30 times higher in rats [34]. The optimal level of ITT to effectively produce sperm is unclear and would be different among individuals because of the different expression levels of androgen receptors (AR), which again mainly localize in Sertoli cells in humans [15], as well as other factors. In fact, Shinjo *et al.* reported that there is no difference in ITT in men after salvage hormonal therapy irrespective of the presence of sperm [35]. At the same time, they showed that pre-treatment basal ITT was lower among men who responded to hormonal treatment than those from whom spermatozoa could be retrieved at the 2nd micro-TESE. This indicates that ITT deficiency is one of the causes of NOA and that this condition can be potentially improved by hormonal therapy. This is of particular importance in Klinefelter syndrome patients, whose serum testosterone levels tend to be lower than those of common NOA cases [36]. Ramasamy *et al.* have shown that men with low baseline testosterone who responded to hormonal therapy with a resultant testosterone levels greater than 250 ng/dL had a 77 % chance of sperm retrieval *vs.* 55 % for men who did not respond to therapy [37]. Serum markers that may be able to predict ITT have been described, including insulin-like factor 3, anti-Mullerian hormone, inhibin B and 17a-hydroxyprogesterone. The correlations between these parameters and ITT should be further investigated to facilitate non-invasive evaluation and monitoring of ITT. At the same time, non-invasive evaluation of AR levels in the testis is important for future research.

Leydig cell-derived growth factors

Several reports have identified Leydig cell-derived growth factors for the proliferation of spermatogonia. Among them, EGF-like growth factors have been well-investigated and play important roles for

spermatogenesis [38-44]. We found that transactivation of the EGF receptor (EGFR), a tyrosine kinase type receptor through the activation of LHR, a G protein coupled receptor, occurred both in MA-10 cells [45] and rat Leydig cell primary culture [46] after hCG stimulation. Representative EGF-like growth factors which bind the EGF receptor include EGF, heparin binding-EGF, amphiregulin, epiregulin, betacellulin and transforming growth factor-α. The presence of all these ligands was confirmed in non-stimulated MA-10 cells and rat Leydig cell primary culture by RT-PCR. EGF, heparin binding-EGF, amphiregulin and TGF-α have been shown to be EGF-like growth factors secreted immediately after hCG stimulation [47]. Additionally, the presence of heparin binding-EGF, amphiregulin and TGF-α are closely associated with spermatogenesis in the human testis [48]. Other groups have reported that EGF, TGF-α and betacellulin stimulate spermatogonial proliferation *in vitro* [43], especially in type A spermatogonia [39].

Role of FSH on spermatogenesis

FSH stimulation of Sertoli cells

Sertoli cells, which localize within seminiferous tubules, directly support spermatogenesis under the regulation of FSH and testosterone. As described above, hormonal dominance of spermatogenesis shifted during evolution from FSH to LH [9], whereas FSH has been considered essential for induction of and qualitative and quantitative maintenance for spermatogenesis [49]. The characteristics of FSHβ KO mice were reported by Kumer *et al*. The serum testosterone level was normal and as a result, normal male external genitalia developed. Additionally, the mice were fertile with decreased testicular volumes and spermatogenesis that was not completely disturbed [50], in contrast with LuRKO mice. The phenotype of FSH receptor KO mice was more disturbed than FSHβ KO mice in terms of genital development and spermatogenesis. However, spermatogenesis was also preserved in this gene-modified animal [51, 52]. Considering all of these observations, it is easy to understand that FSH *per se* is not necessary for spermatogenesis and that the main role of FSH is to increase the quantity of sperm produced in synergy with ITT. ITT also up-regulates Sertoli cell AR and enhances the function of Sertoli cells [15]. In the treatment of male hypogonadotropic hypogonadism, pre-treatment with recombinant human FSH (rhFSH) enhances final spermatogenesis compared to the classical treatment regimen, which started with hCG [53]. This indicates that proliferation of spermatogonia induced by FSH enhances spermatogenesis. A number of studies using gonadotropin-deficient animals have also shown that FSH alone is not able to complete spermatogenesis, except for a small increase in the number of spermatogonia and premeiotic spermatocytes [18]. FSH stimulates prenatal and prepubertal proliferation of Sertoli cells and determines their final cell number, which affects the size of the seminiferous tubules and testis. In adults, FSH stimulates mitotic and meiotic DNA synthesis in spermatogonia and preleptotene spermatocytes and acts as a survival factor for these premeiotic germ cells by acting on Sertoli cells [54]. By stimulating these proximal steps, FSH passively but not directly stimulates the meiotic steps and regulates spermatogenesis.

There are numerous reports on the role of Sertoli cell-derived factors that support spermatogenesis including androgen binding protein, transferrin, ceruloplasmin, plasminogen activator, TGF-α, TGF-β, transferrin, IL-1α, insulin-like growth factor inhibin B and anti-Mullerian hormone. The roles of these factors on spermatogenesis need further investigation.

FSH therapy for spermatogenic disorders

Compared to hCG there are several trials using FSH, classically using hMG and more recently rhFSH. This is because stimulation of Sertoli cells has been believed to contribute more directly to restoration of spermatogenesis than hCG alone because of the localization of Sertoli cells. Most of the results of FSH therapy have been achieved with men who have oligoasthenozoospermia (OAT) not NOA. Nonetheless, the efficacy of FSH therapy for treatment of OAT remains controversial [55-57]. Several studies have evaluated the efficacy of FSH administration for men with OAT with resultant improvements in sperm parameters, whereas other studies have not shown any significant effect. There are several opinions regarding who would benefit from FSH treatment: 1) oligozoospermic men with normal serum FSH levels [57], and 2) FSH receptor genotype men with at least one serine in position 680 who had a significant increase in sperm parameters [58]. Most important, the influence of technology for female infertility treatment such as ICSI complicates the results of male infertility treatment (effect-modifying factor) [55].

An explanation for the poor outcome of FSH treatment is that the doses have been pharmacologically too low (approximately 75-150 IU, three times a week). Paradisi et al. [59, 60] and Ding et al. [61] conducted placebo-controlled studies with a high dose of rhFSH (300 IU every other day for 4-5 months) instead of the standard dose of 75 IU every other day. The results included significantly increased sperm counts and pregnancy rates, suggesting that FSH is regarded as necessary for quantitative improvement of spermatogenesis [59, 60]. Furthermore, Tsametis et al. demonstrated that a dose of up to 300 IU of rhFSH is not sufficient to increase either inhibin B or AMH serum concentrations and suggested that more prolonged administration (presumably multiple injections over a period of two to three weeks) is necessary to achieve clinically meaningful inhibin B stimulation [62]. Paradisi et al. recommended a duration of treatment of at least 4 months to address all stages of a new full spermatogenetic cycle, which lasts approximately 72–75 days [55, 63].

Establishment of gonadotropin therapy for NOA men

The primary difference between OAT and NOA is that the former is a quantitative spermatogenic disorder, while the latter is a qualitative disorder. Based on the approximate 40-60 % sperm retrieval rate from NOA, half of NOA patients have some quantitative disorder, however, the majority of NOA pathophysiology involves qualitative damage at different spermatogenic steps ranging from the Sertoli cell to late maturation arrest. To date, the ability of hormonal therapy to increase sperm production in men with NOA has never been demonstrated in a randomized controlled trial. However, the information obtained from gonadotropin therapy for OAT would at least in part apply to NOA cases.

Due to high gonadotropin levels in men with NOA, medical therapies including hormonal treatments have been believed to be ineffective for improving spermatogenesis because gonadotropin receptors are usually down-regulated. One mechanism of hormonal treatment involves optimizing ITT levels because many men with NOA have low testosterone levels and an abnormal testosterone to estradiol ratio (T/E_2 ratio); however, an increase of E_2 was inevitable because of elevated testosterone. Hussein et al. reported the usefulness of clomiphene citrate as the initial treatment prior to micro-TESE [21]. Reifsnyder et al. also reported a partial effect of testosterone optimization on micro-TESE outcomes following treatment with clomiphene citrate, hCG or aromatase inhibitors. Ramasamy et al. reported that Klinefelter syndrome, men with low baseline testosterone levels who responded to medical therapy to enhance testosterone production with resultant testosterone levels greater than 250 ng/dL had a 77 % chance of sperm retrieval versus 55 % for men who did not respond [64]. These observations suggest that men who can produce increased testosterone levels may also produce increased levels of the other growth factors secreted from Leydig and Sertoli cells [48].

We conducted a preliminary trial of gonadotropin therapy, called salvage hormonal therapy by recruiting men who could not produce sperm by micro-TESE [65]. In spite of high gonadotropin levels, the serum testosterone levels of all of the men were increased after high dose hCG treatment (5,000 units three times a week). The majority of men showed decreased endogenous gonadotropin secretion due to the negative feedback mechanism from increased serum testosterone. Intratesticular hormonal environment is unique because there is a simultaneous "FSH resetting" in the Sertoli cells and an extremely high ITT level from supraphysiologically stimulated Leydig cells [35]. In men with NOA, the relative pulse amplitude of LH secretion was decreased compared to men with normal spermatogenesis [65], indicating that the acute LH/hCG effect in men with NOA was impaired. This provided a rationale to give high dose hCG to intermittently but not to continuously stimulate testosterone secretion in men with NOA. By giving rhFSH for an additional 3 months, 150 IU three times a week, both Sertoli and Leydig cells begun to be stimulated for 2 months. Afterward, the second micro-TESE was effective in 19 % of men who received this salvage hormonal therapy [65].

However, no precise mechanism has been still shown regarding to the involvement of these dynamic hormonal change and restoration of the spermatogenesis. Because histological data have shown that men with hypospermatogenesis or late MA are likely to respond to hormonal treatment [65], spermiogenesis, a step requiring high ITT levels, can be, at least in part, stimulated by hormonal therapy. That hypospermatogeneis and late MA histologies are likely to respond an intervention has been also shown in varicocele repair in men with NOA [66, 67]. Taken together these

observations, the other factors, such as attenuation of oxidative stress and heat damage, improvement of testicular microcirculation, are potentially involved in case of salvage hormonal therapy. Actually the combination of clomiphene citrate and an anti-oxidant could significantly improve sperm count and motility in men with oligozospremia [68], indicating that restoration of spermatogenesis *per se* enhanced testicular oxidative stress and attenuation of it may be beneficial for spermatogenesis. One important criticism, however, is the quality of first micro-TESE. This is because the SRR is likely to be higher if performed at a high volume center [69]. Furthermore, there is a distinct SRR learning curve for individual surgeons [70], affecting different results of salvage hormonal therapy among the institutions. A multi-institutional prospective study by urologists with sufficient micro-TESE experience to validate this protocol has shown a 10 % SRR from the second micro-TESE following the salvage hormonal therapy regimen [71]. The effectiveness of salvage hormonal therapy should be further validated because percentage of hyposoermatogenesis and late MA and surgeons' and embryologists' experiences may be strongly affected on sperm retrieval.

Summary

Hormonal therapy in men with NOA has not been discussed deeply for a long time because infertility treatment can be performed using ejaculate sperms. The era has been changed after the widespread use of micro-TESE and ICSI, which has enabled a chance to retrieve sperms in men with NOA. In other words, an end-point of hormonal therapy is a presence of single sperm in the testis but not large amount of them in the ejaculate. The informations regarding to the cross-talk between Leydig/Sertoli cells and germ cells is still limited and should be further investigated along with the development of reproductive technologies. In addition, we have many opportunities to use human testicular samples which can be obtained at micro-TESE. Informations obtained from the experience of salvage hormonal therapy and further basic informations regarding to male reproductive endocrinology will be able to open a window to establish novel hormonal therapies against NOA.

Acknowledgement

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