Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone

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Evaluation of testicular functions (production of sperm and androgens) is an important aspect of preclinical safety assessment and testicular toxicity is comparatively far more common than ovarian toxicity. This chapter focuses (1) on the histological sequelae of disturbed reproductive endocrinology in rat, dog and nonhuman primates and (2) provides a review of our current understanding of the roles of gonadotropins and androgens. The response of the rodent testis to endocrine disturbances is clearly different from that of dog and primates with different germ cell types and spermatogenic stages being affected initially and also that the end-stage spermatogenic involution is more pronounced in dog and primates compared to rodents. Luteinizing hormone (LH)/testosterone and follicle-stimulating hormone (FSH) are the pivotal endocrine factors controlling testicular functions. The relative importance of either hormone is somewhat different between rodents and primates. Generally, however, both LH/testosterone and FSH are necessary for quantitatively normal spermatogenesis, at least in non-seasonal species.

Background

Evaluation of testicular function is an important and compulsory aspect of preclinical safety assessment. Testicular toxicity is comparatively far more common than ovarian toxicity and histological evaluation of the testis is an integral part of regulatory toxicity studies. This article aims at providing a concise overview on the testicular histology sequelae of endocrine deficiencies and imbalances as deemed relevant for drug development and safety assessment studies. Specifically, rodent (with focus on rat), dog and nonhuman primate (NHP) are being considered. No attempt is made to distinguish between different rat strains or different dog breeds. With regard to NHP models, we focus on macaques—cynomolgus monkey (long-tailed macaque; *Macaca fascicularis*) and rhesus monkey (*Macaca mulatta*). The minipig is entering safety assessment as another potentially relevant animal model but information of endocrine control and effects of

endocrine deficiencies on spermatogenesis is very scarce and for that reason, the minipig is not presented in this review. We also provide an extensive review on the postnatal endocrine regulation of spermatogenesis in the rodent, NHP and human and the current concepts around the relative roles of the key reproductive endocrine factors.

Hypothalamus-Pituitary-Testis Circuit

The endocrine control of spermatogenesis is governed by the neuroendocrine activity along the hypothalamic-pituitary-testicular axis. In the rodent, this is manifest immediately following birth resulting, in rapid succession, in (1) spermatogonial differentiation, (2) completion of first wave of spermatogenesis and (3) puberty into adulthood by the 3rd month of life.¹ In contrast, in higher primates including man, 3 distinct phases, lasting from months to years, of postnatal activity along the hypothalamic-pituitary-testicular axis can be traced before adulthood.^{2,3} The first of these is the neonatal-infantile phase that exhibits adult-like activity along the hypothalamic-pituitary-testicular axis but, notably, absent spermatogenic activity. The second is the protracted juvenile/childhood phase when the hypothalamic-pituitary-testicular axis is quiescent. In the final phase, the hypothalamic-pituitary-testicular axis activity is reinitiated accompanied by pubertal initiation of spermatogenesis leading to normal adult testicular functions.^{2,3}

The pivotal central, hypothalamic signal to the pituitary is the decapeptide gonadotropin-releasing hormone (GnRH) that is characteristically secreted in robust pulsatile manner and acts via the transmembrane GnRH receptor. In turn, the 2 major endocrine signals to the testis, originating from the pituitary gonadotropes that bear GnRH receptors, are the distinct heterodimeric glycoprotein hormones FSH and LH. Typically 70–90% of gonadotropes express both the common α and specific β subunits of the gonadotropins and LH and FSH may be found in the same secretory granules,^{4–8} which by a process of exocytosis deliver the gonadotropins to the peripheral circulation. The gonadotropins are secreted in a pulsatile fashion in response to GnRH. Relatively, and in general, the pulsatile LH release is robust and similar to that of GnRH while the pulsatile release of FSH is rather sluggish.

At the level of the testis, the two gonadotropins, FSH and LH, mediate their actions via specific transmembrane receptors,

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FSH-R and LH-R, respectively. Predominantly, FSH-R is expressed in the Sertoli cells within the seminiferous cords/tubules whereas LH-R is expressed in the interstitial Leydig cells. Both FSH (directly) and LH (indirectly via testosterone-androgen receptor [AR]) exert their actions on spermatogenesis mainly through the regulation of Sertoli cell factors.

In response to the gonadotropins, 2 major endocrine signals are produced from the testis. These are the steroid hormone testosterone, produced by the Leydig cells in response to LH signaling and secreted also in a pulsatile fashion, and the non-steroidal hormone inhibin, produced by the Sertoli cells in response to FSH signaling and secreted in a non-pulsatile manner. Interestingly, evidence from the rhesus monkey suggests an inhibitory role of testosterone (T) on inhibin B secretion.⁹ Together, these gonadal hormones are the major feedback signals that maintain the physiological operation of the hypothalamic-pituitary axis.

It must be reiterated here that, in general, the role of gonadotropins during puberty is primarily to establish the adult cohort of Sertoli, Leydig and stem germ cells and their functions that will eventually lead to normal spermatogenesis and sperm production. Therefore, hormone deprivation during this phase will naturally affect the normal scrotal descent (where applicable) and development of the adult testis. In contrast, in the adult, the effects of hormone deprivation are essentially on the germ cells composition via functional impairments in the somatic cells, particularly the Sertoli cells.

The foregoing complexity of interactions along the hypothalamic-pituitary-testicular axis serves as a reminder that the processes of initiation and maintenance of normal spermatogenesis are prone to impairments, due to toxic effects, not just directly at the level of the testis but at a multitude of other, indirect points along the hypothalamic-pituitary-testicular axis.

Role of FSH

Rodents

Classical studies of the effects of FSH have utilized hypophysectomized or GnRH immunized rats treated with exogenous FSH preparations including recombinant FSH treatment. Animal models such as the GnRH-deficient hypogonadal (*hpg*) mouse, hypophysectomized rat or GnRH neutralized rat treated with FSH have shown that FSH increases germ cell numbers by several fold by increasing the complement of spermatogonia and spermatocytes¹⁰⁻¹⁵ but FSH was unable to generate spermatids in the absence of androgen¹⁴⁻¹⁶ consistent with studies of hypophysectomized rat in which FSH-stimulated post-meiotic germ cell development was inhibited in the absence of androgen signaling.^{17,18}

Studies of the FSH β or FSH-R knockout mouse models (FSH β KO, FSHRKO) suggest that FSH is not essential to maintain fertility.¹⁹⁻²³ Male FSH β KO mice have normal sexual development with decreased testicular volume associated with decreased number of Sertoli cells and sperm but are fertile. Transgenic *hpg* mice that express FSH can support spermatogenesis through the completion of meiosis²⁴ but not complete germ cell maturation independent of testosterone. FSHRKO mice also

have fewer Sertoli cells, small testes but the mice are fertile.^{20,21,25-28}

Together, these findings indicate that, in rodents, while FSH is essential for the pubertal development of full complement of Sertoli cells, the absence of this gonadotropin during pubertal development affects spermatogenesis only quantitatively resulting in decreased sperm output in the adult.

Nonhuman primates and human

Because the postnatal endocrine control of spermatogenesis in nonhuman primates is similar to that of humans, studies of nonhuman primates have provided a suitable surrogate for the physiological actions of FSH in the human testis. In both man and nonhuman primate, 2 types of undifferentiated spermatogonia (A-dark and A-pale) are present regardless of the activity along the hypothalamic-pituitary-testicular axis while the appearance of B spermatogonia signals spermatogonial differentiation and initiation of spermatogenesis. Studies of immunization of adult monkeys against FSH (but with normal testosterone levels) have shown a 50% reduction in the transition of spermatogonia into spermatocytes.²⁹ Men immunized with ovine FSH had antibodies that neutralized FSH bioactivity and sperm counts were reduced.³⁰ FSH deprivation in monkeys appears to inhibit spermatogonial proliferation and their transition to spermatocytes.³¹ On the other hand, the number of spermatogonia was increased after FSH stimulation of intact monkeys or FSH depleted monkey models.³²⁻³⁴ FSH treatment of prepubertal monkeys increased the number of Sertoli cells and initiated spermatogenesis resulting in the production of B spermatogonia and spermatocytes.^{35,36} Together, these data support the idea that, in men and nonhuman primates, FSH acts through the Sertoli cell to facilitate the production and/or survival of spermatogonia and their transition into spermatocytes.

Whether FSH is essential to maintain fertility in men has been extensively analyzed.³⁷ Azoospermia has been a consistent finding in men with loss of function mutations in FSH β , although in one of these subjects circulating testosterone levels were low and virilization at the expected time of puberty did not occur.³⁸⁻⁴⁰ Studies of men with a monotropic deficiency of FSH of unknown etiology and normal virilization and circulating testosterone levels are also worthy of note since several of these individuals have been reported to be infertile.⁴¹⁻⁴³ Moreover, in 2 of these men treatment with either human menopausal gonadotropin (hMG), which has FSH activity, or FSH itself was associated with either the initiation of spermatogenesis or with fertility.^{42,43}

In contrast to FSH β mutations, 5 subjects with mutations in the FSH-R, that greatly reduced incorporation of the mutant protein into the membrane, were reported to be fertile with testes that were smaller than normal and oligospermia.⁴⁴ However, circulating FSH levels were elevated in these subjects, and residual FSH signaling by the mutated receptor cannot be excluded.

Physiological evidence for improved spermatogenic capacity due to endogenous excess of FSH secretion is demonstrated in studies of unilateral orchidectomy of the adult monkey.^{45,46} Removal of one testis results in 50% decrease in inhibin B production and, as a consequence of this decreased feedback signal, a

dramatic and sustained increase in FSH level occurs. This, in turn, results in a significant compensatory hypertrophy of spermatogenesis in the remaining testis particularly by increased B spermatogonia and eventual spermatid numbers.⁴⁶ The foregoing study indicates that, in higher primates, normal spermatogenesis is not operating at maximal capacity.⁴⁶ That this situation may also exist in the human is supported by a recent study.⁴⁷

Role of LH/testosterone

It is generally recognized that AR signaling in germ cells is not required for spermatogenesis and therefore the cell biology underlying the action of LH to stimulate spermatogenesis is complex involving several somatic cell types and a sequence of indirect signaling pathways. The foregoing notion is based on (1) the finding that murine germ cells from animals in which AR has been deleted are capable of spermatogenesis when transplanted into a recipient testis expressing AR⁴⁸ and (2) the absence of compelling/consistent evidence for AR expression by germ cells.^{11,49-55} AR is expressed in several somatic cell types, namely the Sertoli cell, peritubular myoid cell, Leydig cell, vascular smooth muscle and endothelial cells.

Evidence for the critical role of the LH-testosterone signaling pathway in initiating and maintaining spermatogenesis has been obtained from several animal models and experimental approaches.^{1,56} Classical studies utilized hypophysectomy to abolish gonadotropin secretion followed by selective replacement with testosterone or LH. More recently, the required hypogonadotropic state for such replacement studies has been achieved by administration of a GnRH receptor antagonist. Contemporary approaches have utilized transgenic animals bearing loss of function mutations of LH β or LH-R and the GnRH-deficient *hpg* mouse. In addition, chemical ablation of adult rodent Leydig cells *in vivo* by ethane dimethane sulfonate (EDS) treatment has been used to examine the effects of loss of testicular testosterone on spermatogenesis.^{1,57}

Rodents

In the adult rodent, withdrawal of gonadotropin (LH and FSH) secretion by hypophysectomy, GnRH receptor antagonist treatment or active immunization against GnRH leads to an arrest of spermatogenesis albeit with the differentiating spermatogonia entering into meiosis.^{12,13,58-68}

In male mice, in which the gene encoding for LH β has been deleted, serum and testicular testosterone levels in animals of adult age (6 weeks) are extremely low and spermatogenesis is not completely initiated. At this age, round spermatids are the most mature germ cell in the testes of these mutant mice.⁶⁹ It should be noted, however, that lack of LH signaling in these animals was associated with impaired testicular descent.⁷⁰

In male mice, in which LH-R has been deleted, serum and testicular testosterone levels in animals of adult age (8 weeks or older) are extremely low and spermatogenesis is not completely initiated but testes are cryptorchid. Round spermatids are the most mature germ cell in the testes of these mutant mice.⁷¹⁻⁷⁵

Precocious initiation of spermatogenesis has been recently reported in mice with a gain of function mutation in LH-R⁷⁶

though, at later ages of 5–6 months, the mice became progressively infertile.

An essential role of LH in driving spermatogenesis in rodents may also be inferred from the findings that mice, in which either FSH β or the FSH-R have been deleted, are fertile.¹⁹⁻²² FSH signaling during the postnatal period, however, is an important determinant of Sertoli cell proliferation and FSH β and FSH-R knockout mice therefore have a reduced testis size and sperm output, as discussed above.

In rodents, the pivotal role of testosterone in maintaining spermatogenesis is underlined by studies of transgenic mice, in which AR has been deleted globally or in a cell specific manner. Mice in which AR has been knocked out prenatally (i.e., ARKO mice) are infertile.⁷⁷⁻⁸² Interpretation of the significance of these findings, however, are confounded by the failure in these mice of the testis to descend into the scrotum due to the absence of AR signaling during prepubertal development. This problem is eliminated by postpubertal ablation of AR in adult mice expressing a tamoxifen inducible Cre recombinase and in these animals spermatogenesis is arrested with a virtual absence of sperm in the epididymis and testis 50 d after deletion of the receptor.⁸³

The major target for testosterone within the testis appears to be the Sertoli cell and peritubular myoid cell (PTM), as evidenced by the finding that in mice selective and independent ablation of AR in these somatic cell types results in infertility that is associated with reduced numbers of spermatocytes and spermatids in scrotally located testes.⁸⁴⁻⁸⁹ In mice with Sertoli cell specific ablation of AR (SCARKO), spermatogonial number is maintained and spermatogenesis appears to be arrested during late meiosis.^{85,86} It must be noted that, in global or Sertoli cell specific AR knockout mice, Leydig cell development and function are impaired, though testosterone production appears to be unaffected.⁹⁰ Interestingly, inactivation of AR in Leydig cells⁹¹ results in infertility with impairment in post-meiotic germ cell development in association with decreased serum testosterone. Specific deletion of AR in vascular endothelial cells and smooth muscle in male mice does not lead to a robust reproductive phenotype.⁹²

Nonhuman primates and human

In the adult monkey, similar to that in the rodent, the withdrawal of gonadotropin (LH and FSH) secretion by hypophysectomy, GnRH receptor antagonist treatment or active immunization against GnRH leads to an arrest of spermatogenesis.⁹³⁻¹⁰² Undifferentiated type A spermatogonia are generally the only germ cells remaining after hypophysectomy in primates.^{100,101,103,103} A similar regression of the seminiferous tubule has been observed when gonadotropin secretion is suppressed with a GnRH receptor antagonist.^{93-98,102}

Inhibition of gonadotropin secretion in normal men in response to chronic (12–20 weeks) GnRH receptor antagonist treatment in combination with testosterone administration, which maintains circulating testosterone levels but not the intratesticular content of the steroid, results in azoospermia in the majority of subjects.¹⁰⁴⁻¹¹³

Figure 1. Testicular histology in control and GnRH antagonist-treated cynomolgus and rhesus monkeys. Upper left: Control; upper right: Following 16 d of treatment—note the lack of B-type spermatogonia compared to control while other cell types are still present; middle left: following 25 d of treatment—same magnification—note lack of B-spermatogonia and depletion of pachytene spermatocytes and round spermatids and reduction of tubular diameter; middle right—same duration of treatment—note retained elongated spermatid heads at the base of the germinal epithelium (arrowheads); bottom left—following 31 d of treatment—note progressive depletion of germ cells, presence of degenerating cells (asterisks) and tubular shrinkage but also presence of elongated spermatids; bottom right—following treatment for approx. 6 months (bar indicates 100 μ m) - note that only spermatogonia (asterisks) and Sertoli cells remain; Sertoli cells are stained for GATA4 (brown) (image courtesy of Dr. Plant TM, unpublished data). B = B-type spermatogonia, A = A-type spermatogonia, PS = pachytene spermatocytes, RS = round spermatids, SC = Sertoli cells Bar in middle right indicates 100 μ m and in bottom left 50 μ m.

Parenthetically, it should be noted that in man and monkey there is heterogeneity in the response to gonadotropin withdrawal induced by steroid or GnRH receptor analog treatment both between individuals and across seminiferous tubules within the same testis,^{93,94,99,109,114-116} Ramaswamy, Marshall and Plant unpublished observations).

Loss of LH secretion or bioactivity due to LH β mutations in man is associated with very low plasma testosterone (but normal to high FSH) and absent puberty. These individuals are

with the steroid.^{35,149}

Testicular Testosterone and Spermatogenesis

That the concentration of testicular testosterone is several folds higher than that in peripheral circulation is well established.^{1,150,151} However, the question of how much of testicular testosterone is required to initiate, maintain or reinitiate normal

hypogonadal and exhibit oligo- or azoospermia but importantly testicular descent occurs.¹¹⁷⁻¹²¹

The phenotypes associated with inactivating mutations of LH-R in the human are far more complicated because the impact of the receptor deficit on sexual differentiation of the external genitalia are more severe and frequently male pseudohermaphroditism and cryptorchidism is observed.¹²²⁻¹³²

In general, the testes of these individuals are characterized by Leydig cell hypoplasia and the seminiferous tubules have been described as immature and with spermatogenic arrest. One patient of particular interest had a normal male phenotype in association with a homozygous deletion of exon 10 of the LH-R. Interestingly, LH signaling was compromised to a much greater extent than that of human chorionic gonadotrophin (hCG), and this patient had delayed/absent puberty and exhibited azoospermia before treatment with hCG.¹²³ As discussed before, interpretation of an essential role of LH in driving spermatogenesis in men with loss of function mutations of either the FSH receptor or FSH β is more problematic.³⁷ As in the rodent, humans bearing loss of function mutations in AR are infertile.^{77,80-82}

Activation of LH- testosterone signaling prior to the onset of puberty, on the other hand, leads to the precocious onset of spermatogenesis. In the human, reports of activating mutations in LH β or LH-R and Leydig cell tumors are shown to be associated with precocious puberty.^{131,133-145} In some cases where histology of the testes was studied, initiation of spermatogenesis has been reported.^{131,146-148} That testosterone alone can initiate spermatogenesis is also shown in studies of the juvenile monkey chronically treated

Figure 2. Percent change (mean $\pm$ SEM, $n = 5$) of testicular size (open symbols) and sperm numbers in the ejaculate (solid symbols) of cynomolgus monkeys treated with GnRH antagonist for 18 weeks. The open triangle indicates testosterone supplementation at doses to provide normal range serum testosterone but not testicular testosterone levels in order to restore ejaculate behavior. Asterisks denote occasion when no ejaculate was obtained. Modified from ref 95.

spermatogenesis is less defined.¹ A major hurdle to resolving this issue is related to another question, namely how accurately can testicular concentration be measured.¹ Moreover, a great deal of variation in testicular testosterone concentrations has been observed within a given species, between animals and between studies.^{1,46,150} Also, it should be noted that various parameters of testicular testosterone content, such as steroid concentration in spermatic vein, testicular vein, interstitial fluid and seminiferous tubule fluid, testicular homogenates and percutaneous fine needle aspirates,^{1,150,151} have been utilized and the relationship between them is incompletely understood.

The testicular testosterone content that is required to initiate spermatogenesis in hypogonadotropic models is remarkably low compared with levels of this steroid in the normal adult testis. In the *hpg* mouse treated with either hCG or increasing doses of testosterone via subcutaneously implanted Silastic capsule of varying sizes that resulted in testicular concentrations of the steroid of approximately 25% of non-*hpg* control mice, qualitatively normal spermatogenesis has been reported.^{152,153} In the hypophysectomized adult rat, exogenous testosterone treatment at various concentrations that produced testicular testosterone levels up to 10–25% of control values were associated with maintenance or restoration of qualitative spermatogenesis.^{58,60,61,63,64,154} Similarly, in pituitary intact adult rats treated with testosterone, alone or in combination with estradiol to suppress gonadotropin secretion and arrest spermatogenesis, exogenous testosterone that produced testicular levels of the steroid at around 20–40% of normal level was able to maintain or restore spermatogenesis qualitatively.^{62–64,155} In the hypophysectomized adult monkey implanted with testosterone capsules immediately following surgery, testicular testosterone levels of approximately 50% were able to qualitatively maintain spermatogenesis.¹⁰¹ Weinbauer et al.⁹⁵ have shown that the spermatogenic suppressive effect of GnRH antagonist was prevented by exogenous testosterone

Figure 3. Flow cytometric analysis of testicular tissue in control and GnRH antagonist-treated cynomolgus monkeys. Animals were treated for 31 d. It is of interest to note that the proportion of HC (haploid spermatids with reduced chromatin staining due to chromatin condensation hence considered to represent elongated spermatids) is increased compatible with retained spermatids and the proportion of 4C (spermatogonia and primary spermatocytes in G2 phase) cells is decreased compatible with a reduced proliferation of differentiated spermatogonia and subsequently spermatocyte numbers. However, the proportion of 2C (mostly spermatogonia and spermatocytes in G1 phase) cells, representing early spermatogonia is unchanged. The 1C cell population comprises haploid round and elongating spermatids. These observations are concordant with those obtained from quantitative histological analysis. Data represent mean $\pm$ SEM of 4–6 animals per group.

treatment that resulted in only 50% restoration of testicular testosterone. These studies indicate that spermatogenesis will appear qualitatively normal, despite significant decreases in testosterone levels.

Interestingly, in a LH-R knockout mouse model, Zhang et al.⁷² noted initiation of spontaneous qualitative spermatogenesis at 12 months of age apparently due to constitutive production of testosterone supporting the idea that very low levels of testicular testosterone are sufficient for maintaining the spermatogenic process. Similarly, in a milder case of LH-R mutation and in a man with loss of function mutation in LH β , oligozoospermia has been noted in the presence of very low levels of serum or, testicular testosterone.^{156,157} Finally, in normal men receiving contraceptive regimen comprised of testosterone and a progestin to induce hypogonadotropism, a residual testicular testosterone content of 2% was associated with a sperm count of ~ 1 million/ml.¹⁵⁸

At sub-physiological contents of testicular testosterone, there is a direct relationship between content of the steroid and spermatogenic output. In the adult rat treated with various lengths of testosterone containing capsules, elongated spermatid number is directly related to interstitial and seminiferous tubular fluid testosterone concentration.¹⁵⁵ A similar direct relationship between capsule number (and presumable intratesticular testosterone content) and elongated spermatids in the absence of FSH was reported in studies of the *hpg* mouse.¹⁵² At physiological testicular testosterone contents, however, the association between steroid content and sperm output appears to be lost. This notion is supported by studies

Figure 4. Testicular histology in rats. Upper left—control; upper right—following 14 d of treatment with GnRH antagonist—note degenerating germ cells (arrows) and vacuoles (asterisks); lower left—following 14 d of treatment with GnRH antagonist—note retained elongated spermatid heads at the base of the germinal epithelium (arrowheads); lower right—long-term endocrine suppression with a 17,20-lyase inhibitor—note that pachytene spermatocytes and round spermatids are still present but reduced in number, while elongating and elongated spermatids are absent. PS = pachytene spermatocytes, RS = round spermatids.

of the adult rhesus monkey, in which endogenous gonadotropin secretion is abolished with GnRH receptor antagonist treatment and immediately replaced by administration of “physiological” doses of recombinant human LH and FSH.³⁴ In this experimental preparation known as a testicular clamp, a selective monotropic increase in LH stimulation of the monkey testis that resulted in a fold increase in circulating testosterone, and presumable that of the steroid content within testis, had no impact on spermatogenesis as assessed

by quantitative morphometry.³⁴ Thus, it would seem reasonable to propose that under physiological conditions the negative feedback control system governing testicular testosterone synthesis and secretion plays no role in regulating sperm output because in the normal testis the level of testosterone is maintained far in excess of the limit that needs to be attained to achieve maximal androgen dependent spermatogenesis. Rather, the purpose of this feedback control system is to maintain circulating testosterone levels at a set point that is optimal for the function of androgen dependent non-gonadal tissues such as muscle, brain and male reproductive tract.

Thus, regardless of species and the nature of the spermatogenic arrest induced by a hypogonadotropic state, appropriate replacement with LH (hCG) or testosterone will restore spermatogenesis, albeit generally at levels below that observed under physiological conditions, in which the testis is also stimulated by FSH.^{58,59,61-64,67,73,74,95,97,100,101,152-154,159,160}

Taking the foregoing findings together, it seems reasonable to conclude that LH signaling plays a pivotal role in the initiation and maintenance of spermatogenesis in mammalian species. However, whether LH signaling alone is sufficient and necessary for these processes in all species, as it is in mice, is much less clear. In the human male, for example, an obligatory role of FSH in the initiation of spermatogenesis at puberty is difficult to unequivocally exclude based on the extant data.

While it is well established that testosterone action is mediated via genomic AR signaling, a more recent advance in our knowledge is the non-genomic mechanism of action of testosterone on spermatogenesis.¹⁶¹⁻¹⁶³ Consequently some intracellular signaling might be impaired, however, whether the final effect of these mechanisms of androgen action on specific compartment(s) of germ cells is similar or distinctly different needs to be established.

Finally, other reviews in the literature on the hormonal control of spermatogenesis^{37,164-169} and the relevance to reproductive toxicology^{170,171} are available for further reading.

For this review, the presentation and examination of testicular histology has been confined to light microscopy and tissue fixation and preparation that typically would apply to protocols for more standardized safety assessment studies. This comprises fixation in formalin (not recommended), Bouin's or modified Davidson (recommended), embedding in paraffin and staining with hematoxylin-eosin or PAS-hematoxylin.

In the NHP, gonadotropin deficiency initially provokes a profound reduction in the number of B spermatogonia^{99,116,172} and this spermatogenic lesion has also been described for gonadotropin-deficient men.¹¹⁰ In the cynomolgus monkeys, this loss of B spermatogonia has been observed within about 2 weeks after initiation of treatments (Fig. 1). In terms of cell numbers, there is also some decrease in the number of A-pale spermatogonia but this decrease is only apparent after enumeration of cell numbers

and cannot be discerned upon qualitative analysis of testicular histology. With continuation of treatment, germ cells beyond the spermatogonial phase (e.g. spermatocytes at all stages followed by round spermatids and then elongating spermatids) will be lost increasingly owing to the lack of supply of new spermatogonia that could develop in to more advanced germ cells. Since B spermatogonia divide mitotically 3-times, every B spermatogonium potentially gives rise to 16 preleptotene spermatocytes. Hence, any depletion of B spermatogonia will trigger a rapid and substantial depletion of subsequent germ cell generations. In fact, within 4–5 weeks of gonadotropin deficiency, the loss of more advanced germ cell becomes readily apparent histologically (Fig. 1).

During prolonged suppression of reproductive hormone support, the spermatogenic process will be interrupted at the level of spermatogonia in the NHP (**Fig. 1**)—there is substantial interanimal variability but complete suppression of spermatogenesis is usually achieved within 8–16 weeks of treatments. **Figure 2** depicts the alterations of testicular size and sperm numbers in

Figure 6. Testicular histology in the dog. Upper left—control; upper right—following acute testosterone depletion—note pronounced loss of germ cells, multinucleated cells (double asterisks) and germ cell with abnormal morphology (asterisk); lower left—following 14 d treatment with a neurokinase inhibitor—note detached germ cells in the lumen or in epithelium (asterisk); lower right—following chronic endocrine suppression—note that only spermatogonia and Sertoli cells are present. Sp = spermatogonia, SC = Sertoli cells.

NHPs during prolonged gonadotropin suppression and during recovery. Within four weeks, testicular size has declined to about 40% of baseline dimensions and the histological appearance of the germinal epithelium is noticeably altered (Fig. 1). Testicular

and in clear contrast to primates, rat spermatogenesis responds quite differently to gonadotropin depletion. For the primary spermatogenic lesion, the more advanced germ cells such as pachytene spermatocytes, plus round spermatids exhibit the

size will decrease to maximally 20–25% of baseline dimensions (Fig. 2) probably representing the physical limit of tissue compaction. In this stage of testicular involution, the germinal epithelium contains Sertoli cells and few A-type spermatogonia (Fig. 1). Sperm numbers in semen samples drop within a month, and after 8–16 weeks of treatment azoospermia has been observed (Fig. 2). Upon cessation of treatment, testis size completely recovered within about 10 weeks followed by sperm numbers about 8 weeks later (Fig. 2). The lag time of 8 weeks of recovery between testis size and sperm numbers is compatible with the entire duration of spermatogenesis (6 weeks from differentiated spermatogonia to testicular spermatids) in the cynomolgus monkey.

Furthermore, endocrine suppression is associated with retention of spermatids that are not released anymore from the germinal epithelium both in monkey and man.^{110,116} The qualitative histological changes (loss of spermatogonia and retention of elongated spermatids) can also be demonstrated in a quantitative manner by testicular flow cytometry of NHP testicular tissue (Fig. 3). Following gonadotropin suppression by a GnRH antagonist, the proportion of elongated spermatids (HC cells, haploid cells) is increased (retained spermatids) while the proportion of 4C cells (spermatogonia and primary spermatocytes in G2 phase) is decreased. Also, cell proliferation studies using 5-bromodeoxyuridine incorporation coupled with flow cytometry, have provided functional confirmation of this effect and demonstrated a reduction of 2C cells (mostly spermatogonia and spermatocytes in G1 phase) that had incorporated 5-bromodeoxyuridine within 16–25 d of GnRH antagonist exposure.¹⁷²

Rodents

Retention of spermatids following endocrine suppression also occurs in rats (for review see O'Donnell et al in this journal volume). Otherwise, however,

earliest signs of cell degeneration (apoptosis) and this effect is highly stage-specific being limited to stage VII/VIII tubules (Fig. 4). In addition step 19 elongated spermatids fail to be released during stage VIII and are retained into later stages (spermatid retention, Fig. 4), due to the fact that spermiogenesis is a testosterone dependent function. These effects are also seen within days of selective elimination of Leydig cells, which are the source of testicular androgens, suggesting that these stages are androgen-dependent (for details on testicular histology see reference 173). Another potential difference between rodent and NHP resides in the observation that in the rat, even after prolonged endocrine inhibition, spermatocytes and most round spermatids are still present in the germinal epithelium (Fig. 4). It is interesting to note that this is also the case in long-term hypophysectomized rats and it has been suggested that there is some residual gonadotropin support originating from the pars tuberalis in such cases. Also, after sustained suppression of gonadotropin and testosterone production (e.g. 17- β lyase inhibitor), spermatids are the major cell type affected resulting in an end stage lesion of tubules containing no elongating spermatids, while round spermatids, spermatocytes and spermatogonia are still present (Fig. 4).

Thus, rodent and primate spermatogenesis respond quite differently to reproductive hormone withdrawal in terms of germ cell type-specific effects and their relation to the spermatogenic stages (Fig. 5). The primary spermatogenic lesion affects different germ cell types (except elongating spermatid retention which is common to both species) and the maximal spermatogenic suppression is more pronounced in NHPs compared to rat.

Dogs

Unlike rodents and primates, less information is available on the relative importance of LH, FSH and testosterone for the spermatogenic process in canines. On the other hand, a number of studies have investigated the sequelae of GnRH agonist treatment on testicular histology and function. Typically, in the dog, gonadotropin deficiency provokes a complete cessation of spermatogenesis within about 2–4 weeks (Fig. 6) and absence of spermatozoa in the ejaculate within 5–7 weeks. Based upon testicular histology, mild spermatogenic effects with some multinucleated cells were observed within 10 d of GnRH agonist treatment followed by complete suppression by day 38 with seminiferous tubules containing spermatogonia/spermatocytes.¹⁷⁴ Giant cell formation and germ cell degeneration occurs within 14 d of endocrine disturbances (Fig. 6). Following prolonged suppression of reproductive hormone secretion, the germinal epithelium contains—besides Sertoli cells—spermatogonia and spermatocytes (Fig. 6) or only spermatogonia (Fig. 6). Following prolonged suppression (e.g. by a GnRH antagonist), only few spermatocytes remain and following cessation of treatment, spermatocytes and spermatids reappear within 3–9 weeks and elongated spermatids within

Figure 7. Testicular histology in the dog. Upper left—following GnRH agonist treatment for about 5 months—note that few spermatocytes (arrows) are the most advanced germ cells; upper right—following 3–9 weeks of recovery—note that round spermatids (arrow) have reappeared; lower left—following another few weeks of recovery and elongating spermatids are present (arrows); lower right—within 3–6 months spermatogenesis is completely restored and mature elongated spermatids are present (arrows). Modified from Goericke-Pesch.¹⁷⁵

9–26 weeks (Fig. 7). Overall, it appears that the spermatogenic response to reproductive hormone deficiency in the dog occurs very rapidly and progresses to an end stage lesion of testicular involution, where the seminiferous tubules contain only spermatogonia and Sertoli cells. In that context the dog appears similar to primates except that the onset of spermatogenic involution occurs comparatively earlier.

GnRH agonists downregulate the pituitary GnRH receptor followed by a decrease in gonadotropin secretion and testicular testosterone production. Downregulation of hormone secretion for approximately 5 months as evidenced by determinations of FSH, LH and testosterone resulted in complete cessation of spermatogenesis—either at the level of spermatogonia (approx. 70% of seminiferous tubules) or at the level of spermatocytes (approx.

Figure 8. Testis size in dogs before and during 25 weeks of treatment with a GnRH agonist (mean $\pm$ SD of 8 animals) and following removal of the GnRH agonist implant (5 animals) for another 25 weeks. Data compiled from Ludwig.¹⁷⁷

Figure 9. Comparison of testis size determinations either by sonography (left panel) or caliper (right panel) with testis weight in the cynomolgus monkey. Size measurements were collected prior to necropsy for comparison with true testis weight. Caliper measurements tend to underestimate testis weight possible since some pressure needs to be applied for consistency of measurements, while following sonographic size assessments, the data appear closer to testis weight.

30% of seminiferous tubules).¹⁷⁵ In another study, after GnRH agonist administration for about 20 weeks, spermatocytes were still present in 11–95% of seminiferous tubules; 176). Testis volume was reduced by 55%. In other instances, testis size reductions of up to 80% have been reported within 16–18 weeks of hormone deficiency¹⁷⁷ (Fig. 8). Prostate size was reduced by approx. 50%. Recovery of spermatogenesis was investigated at different intervals: spermatocytes re-appeared within 3 weeks, round spermatids within 3–9 weeks, elongating spermatids within 3–9 weeks and elongated spermatids (complete spermatogenesis) within 9–24 weeks (Fig. 7).¹⁷⁴ Recovery of testis size occurred within 24–27 weeks (Fig. 8) followed by sperm numbers within 29 weeks. Interestingly, during the recovery period, the blood levels of gonadotropins and testosterone were comparable irrespective of whether spermatogenesis had progressed to the level of spermatocytes or round or elongating/-ed spermatids—suggesting that the first rebound of hormone secretion¹⁷⁸ triggered the spermatogenic process in large part of the testis. This would be roughly consistent with the 56–93 d duration of dog spermatogenesis.¹⁷⁹

Testicular size determination

Various approaches are available for repeated determination of testicular size during various study phases e.g., by orchidometer, caliper or sonography.^{95,177,180} Orchidometers provide a simple approach but the accuracy is limited by the given orchidometer size intervals. Calipers have been used frequently and provide a rather precise method if used by a single experienced investigator while the inter-individual differences can be substantial. The reason being that the value for the testicular width is squared in the computational formula—hence even rather small differences in the width assessment can result in comparatively large differences. Ultrasound assessment of testicular size appears to be more accurate and precise than caliper measurements in that caliper measurements tend to underestimate testis weight while

sonographic determinations appear rather close to true testis weight (Fig. 9). For the ultrasound approach, typically the largest testicular diameter is used and the organ volume then calculated by the instrument. Since sonographically determined testis size correlated well with testis weight at necropsy, measurements of testis size by ultrasound at baseline and at necropsy (and intervals inbetween) can be useful to determine longitudinal changes in testis size and to assess testis and “weight” prior to study start. Another advantage of ultrasound approach is that the frozen picture can be stored and printed thus allowing documentation and potentially further analysis at a later timepoint. Also, testicular texture is available from these pictures. Therefore, ultrasound determination of testicular size appears to provide advantages over the use of orchidometers and calipers.

Conclusions

1. Rodent and primate spermatogenesis respond quite differently to reproductive hormone withdrawal in terms of germ cell types affected and their relation to the spermatogenic stages. The primary spermatogenic lesion affects different germ cell types (except elongating spermatid retention which is common to both species) and the maximal spermatogenic suppression is more pronounced in NHPs compared to the rat.
2. The spermatogenic lesion in response to reproductive hormone deficiency in the dog occurs very rapidly and progresses to complete testicular involution, where the seminiferous tubules contain only spermatogonia and Sertoli cells. In that context the dog appears similar to primates except that the onset of spermatogenic involution occurs comparatively earlier.
3. LH/testosterone and FSH are the pivotal endocrine factors controlling testicular functions. While the relative importance of either hormone appears somewhat different between rodents and primates, generally, however, both LH/testosterone and FSH are necessary for quantitatively normal spermatogenesis, at least in non-seasonal species.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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