CONSEQUENCE OF PREMATURE AND CHRONIC LUTEINIZING HORMONE RECEPTOR ACTIVATION ON TESTICULAR SPERMATOGENIC CELL DEVELOPMENT

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CONSEQUENCE OF PREMATURE AND CHRONIC LUTEINIZING HORMONE RECEPTOR ACTIVATION ON TESTICULAR SPERMATOGENIC CELL DEVELOPMENT

by

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the field of Molecular, Cellular, and Systemic Physiology

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TITLE: CONSEQUENCE OF PREMATURE AND CHRONIC LUTEINIZING HORMONE RECEPTOR ACTIVATION ON TESTICULAR SPERMATOGENIC CELL DEVELOPMENT

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Luteinizing hormone (LH), one of the two gonadotropin hormones released from the anterior pituitary gland, binds to its receptor (LHR) in the gonads to stimulate steroid hormone production, in addition to ovulation and gametogenesis. Mutations of the receptors amino acid sequence have the ability to either constitutively activate or inactivate it. All activating mutations result in male-limited precocious puberty. Males with this condition undergo puberty around 4 years of age, and have a premature elevation in testosterone levels and premature skeletal development. In order to understand how chronic ligand-mediated activation of the LHR affects gonadal development and function, a mouse model expressing a yoked hormone-receptor (YHR) complex, engineered by covalently linking the hormone human chorionic gonadotropin to the rat LHR, has been studied. YHR⁺ males have prepubertally elevated testosterone and decreased gonadotropin levels, smaller testis, and smaller average seminiferous tubule diameters when compared to wild type (WT) animals. In a preliminary breeding study it was shown that YHR⁺ males were sub-fertile. Based the phenotype exhibited by the YHR⁺ mice, it was hypothesized that increased levels of testosterone in addition to
decreased gonadotropin hormone levels in neonatal and prepubertal mice that results from premature activation of the luteinizing hormone receptor causes spermatogenesis to be impaired. The first objective of this study was to determine if there was a difference in the testicular germ cell and Sertoli cell populations in the WT and YHR⁺ animals using flow cytometry and systematic Sertoli cell counting, respectively. There was no difference in the Sertoli cell population between YHR⁺ animals and WT controls, but there were significantly fewer total germ cells in YHR⁺ animals at 10 days and from 4 weeks through adulthood. The second objective was to calculate the daily sperm production in the testis and epididymis of WT and YHR⁺ animals in order to determine if there is a further decrease in the total sperm count due to an epididymal dysfunction. Interestingly, there were significantly fewer sperm calculated in the caput/corpus region of the epididymis in YHR⁺ males at 12 weeks of age, but not in the testis and cauda epididymis. Furthermore, the daily sperm production in WT and YHR⁺ mice at 16 weeks of age were not significantly different. The final objective was to determine if the decrease in germ cells observed in YHR⁺ animals is the result of decreased proliferation or an increase in either germ cell or Sertoli cell apoptosis. Quantification of germ cell and Sertoli cell proliferation revealed no significant difference between the WT and YHR⁺ animals. Similar findings were found after quantification of apoptotic germ cell and Sertoli cells. Taken together, these data suggest that premature elevation in testosterone and persistently lower levels of circulating follicle stimulating hormone (FSH) are affecting Sertoli cell function, which is causing a reduced germ cell to Sertoli cell ratio in the YHR⁺ mice. These data suggest that the decrease in testis weight and seminiferous
tubule diameter in YHR\textsuperscript{+} mine is due to a decrease in germ cell rather than Sertoli cell number.
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LIST OF ABBREVIATIONS

Luteinizing Hormone ................................................................. LH
Luteinizing Hormone Receptor ................................................. LHR
Follicle Stimulating Hormone .................................................. FSH
Follicle Stimulating Hormone Receptor ..................................... FSHR
Thyroid Stimulating Hormone ................................................... TSH
Gonadotropin Releasing Hormone ............................................ GnRH
Yoked Hormone-Receptor ......................................................... YHR
Wild Type .............................................................................. WT
Yoked Hormone-Receptor Expressing Mouse ............................ YHR⁺
G Protein-Coupled Receptor ..................................................... GPCR
Cyclic Adenosine Monophosphate ............................................. cAMP
Protein Kinase A ................................................................. PKA
Familial Male-Limited Precocious Puberty .............................. FMPP
Hypogonadal Mouse Model .................................................. $hpg$
Follicle Stimulating Hormone Knock Out Mouse Model .......... FSHKO
Follicle Stimulating Hormone Receptor Knock Out Mouse Model .... FSHRKO
Sertoli Cell Androgen Receptor Knock Out Mouse Model ........ SCARKO
Testicular Feminized Mouse Model ........................................... tfm
Diaminobenzidine .............................................................. DAB
CHAPTER ONE
BACKGROUND AND SIGNIFICANCE

Spermatogenesis is a complex cyclic process that takes place within the seminiferous epithelium of the testis and encompasses the division of diploid spermatogonia, meiosis of spermatocytes, differentiation of haploid round into elongated spermatids, and the release of mature spermatozoa into the lumen. In order for reproductive competence to be established, proper communication and signaling between the different cells of the testis is essential. The goal of this study is to determine how premature and persistent activation of the luteinizing hormone receptor impairs germ cell development.

Gonadotropin Hormones and Receptors

Luteinizing hormone (LH) is a member of the heterodimeric family of glycoprotein hormones. This family of glycoproteins also includes follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG), and thyroid stimulating hormone (TSH). All the members of the heterodimeric family of glycoprotein hormones share a common $\alpha$-subunit while the $\beta$-subunit confers hormone specificity (2). LH and FSH are secreted from the anterior lobe of the pituitary upon stimulation from gonadotropin releasing hormone (GnRH). LH and FSH travel to the testis where they bind to their receptors on the Leydig and Sertoli cell, respectively, and exert their effect. Unlike FSH, LH acts indirectly on the qualitative and quantitative production of sperm. Binding of LH to its receptor on the Leydig cell, results in the production of testosterone, which binds to
the androgen receptor in the cytoplasm of the Sertoli cell and influences spermatogenesis. In addition to androgen, FSH is required for quantitatively normal sperm production, and will dictate the reproductive competence in males. In order to regulate the appropriate hormone levels circulating throughout the body testosterone will negatively regulate itself at the hypothalamic level by inhibiting GnRH, and at the pituitary level by inhibiting the release of LH. The circulating concentration of FSH is under the regulation of inhibin, and in contrast to testosterone, inhibin regulates the release of FSH at the pituitary (figure 1).

![Figure 1. Schematic Representation of the HPG Axis In Males.](image-url)
The LH receptor (LHR), and the FSH receptor (FSHR) are G protein-coupled receptor (GPCR) that have seven hydrophobic transmembrane alpha helices connected by three extracellular and three intracellular hydrophilic loops. Upon binding of the appropriate ligand to the large extracellular domain, the receptor will undergo a conformational change that allows the release of the stimulatory G-protein (Gαs) subunit that is bound to the receptor internally. Activated LHR leads to production of cyclic AMP (cAMP), which will activate cAMP dependent protein kinase A (PKA). The active PKA catalytic subunits are released following cAMP binding to the PKA regulatory subunit, and the active subunits are able to phosphorylate targets that result in transcription and translation of various enzymes needed for androgen production and for the proper development of germ cells during spermatogenesis. High ligand levels have been shown to activate the inositol phosphate signaling pathway, resulting in elevated intracellular Ca^{2+} (3, 4).

In order to maintain the proper testis physiology and quantitatively normal germ cell production, normal signaling through the receptor must occur. Mutations in LHR occur in two forms, activating and inactivating (5). Inactivating mutations are present throughout the receptor (3). For humans that have inactivating mutations, males exhibit impaired sexual differentiation, while females are infertile. Such alterations can result from impairment in the trafficking of the receptor to the cell surface, or an inhibition in ligand binding (5). Activating mutations have been found to be localized to the transmembrane helices and intracellular loops encoded by exon 11 (3). These mutations result in sporadic of familial male-limited precocious puberty (FMPP). FMPP- associated gain of function mutations of the LHR allow the receptor molecule to activate the Gαs
subunit causing testosterone to be produced in the absence of ligand binding. The most common mutation is the replacement of aspartic acid for glycine at position 578 of the sixth transmembrane helix, which is believed to destabilize the inactive conformation (6). Females with an activating mutation have no apparent phenotype, while males begin to undergo puberty anywhere from birth to 3 or 4 years of age. The males have been characterized as having elevated testosterone levels, premature secondary sex characteristic development, Leydig cell hyperplasia, and premature skeletal development (7). Typically, these males are treated with an inhibitor of the cytochrome P450 enzyme, which prevents the production of testosterone until they each an age that testosterone production no longer has a detrimental effect on skeletal development (8). The abnormalities that result from mutations in either the reproductive hormones or their receptor emphasize the importance of proper communication between higher brain centers and the gonads in order to achieve full reproductive competence.

**Spermatogenesis**

Spermatogenesis is a complex cyclic process, in which germ cells undergo mitotic division (spermatogonia), meiosis (spermatocytes), and morphological maturation (spermatids) in a delicately regulated spatiotemporal fashion within the seminiferous epithelium (9). The dynamic events that take place within the seminiferous tubules of the testis occur in close contact with the Sertoli cells. The specialized environment that is required for the successful development of spermatozoa is achieved by the unique morphology of the Sertoli cells, which line the seminiferous tubules, and the formation of the blood-testis barrier (10, 11). What makes the Sertoli cell different from other cells is
its ability to support the migration of a population of cells that proliferate at the base of the tubule and progressively migrate toward the lumen of the tubule as the cells differentiate and mature (10).

The complex events that occur during spermatogenesis can be summarized into four major elements: (1) stem cell renewal by the process of mitosis, (2) stem cell amplification by mitosis and differentiation, (3) reduction of chromosomal number by meiosis, and (4) the transformation of a conventional cell into the complex structure of the spermatozoon by a series of changes involving no further cell division, but rather, a metamorphic process termed spermiogenesis (figure 2) (10). Upon completion of spermatogenesis, mature spermatids are released from the Sertoli cell into the lumen of the seminiferous tubule and move through the rete testis where they then enter the epididymis. The sequence of events that results in the formation of mature spermatozoa from precursor cells takes 34-35 days for the mouse (12). Germ cell development is not a randomly distributed event within the seminiferous tubule, but rather it is arranged in a strictly defined cellular association (13). More specifically, a particular association of germ cells is referred to as a “stage,” and the number of stages in a particular species is defined by the number of morphologically recognizable germ cells. In the mouse there are 12 stages (14). The stages occur in a consecutive wave along the length of the seminiferous tubules. For example, in the mouse, a Sertoli cell will support germ cells that progress from stage I-XII before they will again begin supporting stage I germ cells. The completion of one series of stages in known as a “cycle” of the seminiferous epithelium (14). The most immature germ cells are the spermatogonia, which include type A (A1-4), intermediate, and type B (15, 16). The stem cell pool for the germ call line
consists of a subset of type A spermatogonia, but the true identity of the stem cell cannot be completely discerned on the basis of morphology or by biochemical means (15, 16). In the mouse, as well as other mammals, both type A and B spermatogonia undergo a series of mitotic divisions to produce a large pool of germ cells available to enter into meiosis. The first meiosis begins with the formation of primary spermatocytes (preleptotene, leptotene, zygotene, and pachytene) (16). During the prophase of the first meiotic divisions the germ cells undergo morphological transitions that allows them to be classified on the basis of their nuclear size and morphology. Replication of DNA occurs during the preleptotene phase, and pairing of homologous chromosomes occurs during the zygotene phase. Cells that have chromosomes that are completely paired are the pachytene spermatocytes (14, 16). The developing germ cells then undergo the first meiotic division to yield secondary spermatocytes, which quickly progress through a second meiotic division, yielding a haploid round spermatid (14). Following the production of round spermatids, spermiogenesis, which is the transformation of a round spermatid into an elongated spermatid, through a series of cytodifferentiative steps (13). The cytodifferentiative steps that make up spermiogenesis include: formation and development of the acrosome and flagellum, condensation of chromatin, reshaping and elongation of the nucleus, and removal of the cytoplasm prior to release of the spermatid from the Sertoli cell (13). The final step of spermatogenesis is spermiation, which involves the removal of the spermatid cytoplasm to yield a streamlined spermatozoon that is capable of motility, retraction of the Sertoli cell away from the spermatid and finally, the release of the spermatid into the seminiferous tubule lumen (13).
During spermatogenesis, the developing germ cells undergo mitosis, meiosis, and structural remodeling into mature spermatozoa.

Production of the appropriate amount of spermatozoa depends on the proper regulation and testes stimulation of LH and FSH. The need for testosterone and FSH in the maintenance of normal spermatogenesis has been firmly established, but both FSH and testosterone act in a stage dependent manner and act at different cellular sites during spermatogenesis in order to optimize the spermatogenic process. The existing dogma is that high levels of intratesticular testosterone are required for the onset, maintenance, and completion of spermatogenesis in adult testis (9). Other studies indicate that both FSH and testosterone are required for qualitative and quantitatively normal spermatogenesis. Studies suggest that FSH stimulates the early events in spermatogenesis, including
spermatogonial proliferation and meiosis, but only testosterone is able to sustain complete spermatid differentiation (17). Interestingly, FSHKO and FSHRKO mice are still able to produce a low number of mature spermatids, but human males that have an inactivating FSHβ mutation are azoospermic (4, 18). Hence, many of the details about the exact role of gonadotropins in spermatogenesis remain unclear. To further confound the topic, even though there is a good understanding of the endocrine factors required for spermatogenesis, little is known about the molecular targets for these factors. For example, it is known that testosterone is absolutely required for spermatogenesis, but few androgen-regulated genes that have known function in spermatogenesis have been identified within the seminiferous tubules (19-21).

Based on information that exists in the literature, a number of key factors regulating testicular cell function have been identified and the mechanisms that underlie pituitary hormone and androgen control of testis development have become more established over the recent years. While it is clear that testosterone is absolutely required for germ cells to progress through the early stages of meiosis, the requirement for FSH is a little less clear because animals that either do not produce endogenous FSH or lack the necessary receptor have been found to be fertile.

**Action of FSH and Testosterone in Sertoli Cells**

To reach full reproductive potential, Sertoli cells must first proliferate, which begins in the fetus and ceases around 15-20 days postnatal (22). The commonly held view is that the population of Sertoli cells is fixed and unmodified by hormonal manipulation after puberty (23). However, new evidence is emerging from gonadotropin
deficient hamsters and humans that show that Sertoli cells retain proliferative capabilities and can re-acquire features that are seen in immature Sertoli cells (24). It is the number of Sertoli cells that determines testicular size, germ cell number per testis, and spermatozoa output. What controls the final Sertoli cell number is the result of a dynamic interplay between androgen and FSH, but it is still relatively unknown what the importance of the interaction of FSH and androgen is (25). For example, the degree of overlap or redundancy that these molecules have in regulating Sertoli cell function and thus spermatogenesis is uncertain, and so it is possible that the ablation of one hormone or its receptor may be compensated by the presence of the other hormone (26). Similarly, the degree to which Sertoli cell function and germ cell development is independent of direct hormonal stimulation is unclear.

The role that each of these hormones play in regulating Sertoli cell function and spermatogenesis has become more clear through the study of animals lacking specific hormones or hormone receptors. These animal models include: the hypogonadal (hpg) mouse, FSH knockout (FSHKO), FSH receptor knockout (FSHRKO), Sertoli cell androgen receptor knockout (SCARKO), and testicular feminized (tfm) animals. From studies that were done on animals lacking FSH or FSHR, it was found that these animals had a significant reduction in Sertoli cell number, but these animals still remain fertile, which indicates that FSH acts primarily to optimize spermatogenesis and germ cell number (27, 28). Although the role that FSH performs in spermatogenesis is controversial, it has been reported that full spermatogenesis is not absolutely dependent upon FSH (29). In FSHRKO mice, the Sertoli cell to germ cell ratio is reduced but not markedly, and although there is a 40% reduction in round spermatids, FSHRKO mice
still produce post meiotic sperm, compared to almost a complete absence of round spermatids in SCARKO animals (26). Thus, although FSH is not required for the production of round spermatids, it is necessary in order for sperm to reach full viability and motility (28).

The hpg model has been useful in elucidating the role that androgen and FSH play in Sertoli cell development because these animals lack GnRH through a natural mutation, and as a result these animals have low levels of circulating LH and FSH (22). Through the use of hpg animals, Sertoli cell function and spermatogenesis have been found to be regulated by the actions of FSH and androgen (29). From the studies that were conducted using hpg mice, it was found that Sertoli cell numbers are normal during fetal development, but low levels of androgen occurring in early postnatal life coincide with a decrease in Sertoli cell number (30, 31). Further support for the importance of androgen comes from studies done with tfm mice, which lack a functional androgen receptor. In these animals, Sertoli cell numbers are significantly lower at birth, and remain significantly lower throughout adulthood (22).

From this we can conclude that androgens appear to be essential for Sertoli cell proliferation during fetal development and throughout the postnatal and pre-pubertal development (22). Consistent with these findings, levels of circulating testosterone, the activity of Leydig steroidogenic enzymes, the production of testicular testosterone, and the total steroid content per Leydig cell are greatest during this period. Therefore, there is a correlation between proper Sertoli cell proliferation and activity during development and in the adult animal that is crucial for normal fertility.
**Epididymal Maturation**

As discussed in the previous section, spermatogenesis provides unique cells that have modified nuclei, a distinct DNA content, and a reduced and highly specialized cytoplasm. Despite these modifications, spermatozoa that leave the testis lack the necessary attributes that are required for survival in the female reproductive tract and fertilization of an oocyte. There is strong evidence showing that the epididymis plays a critical role in transforming spermatozoa that leave the testis from immotile cells that are unable to fertilize an oocyte, into fully matured cells that are capable of swimming, recognizing an oocyte, and fertilizing the oocyte. Under normal conditions, the acquisition of these functions is completed by the time sperm enter the proximal cauda epididymis (32). In addition to sperm maturation, the epididymis also plays an important role in sperm transport, concentration, protection, and storage (32). What makes epididymal development so unique is that at the end of spermatogenesis, during spermiation, most of the cytoplasm and the cell organelles that are present in the developing spermatozoa are phagocytized by the Sertoli cell. Because of the re-absorption of the cytoplasmic droplet, the spermatozoa have virtually no transcriptional activity at the late elongated spermatid stage, which occurs generally several days before the completion of spermatogenesis. Thus, because the spermatozoa have virtually no biosynthetic capability, fluids bathing the cells are responsible for sperm maturation and the epididymal epithelium must provide the necessary biocatalysts or ions. Also important during epididymal transit are the degradation of constituent proteins and the removal of defective spermatozoa, which is regulated by a ubiquitin degradation/recycling system (33). The maturational modifications that occur during
epididymal transit are necessary for spermatozoa to become fertilization competent and to be stored safely.

The epididymis is divided into three functionally distinct regions: caput, corpus, and cauda. The caput region contains most of the efferent ducts in addition to the proximal portion of the epididymal duct (34). Collectively, the efferent ducts and the epididymis have four functions: removal of water conveying spermatozoa distally from the seminiferous tubules, transport of spermatozoa distally to the ductus deferens by smooth muscle contractions; maturation of spermatozoa, and storage and maintenance of fertile spermatozoa. Typically, a spermatozoon is transported through the caput and corpus epididymis in 2-4 days, and the cauda epididymis in 6-14 days (35, 36). Most of the differences in transit time among species occur in the cauda (32). When spermatozoa enter the epididymis, they are propelled by testicular fluid pressure and the beating of ciliated cells of the efferent ducts. Once the spermatozoa reach the epididymis, the epididymal epithelium is lined with immotile cilia and there is a massive uptake of fluid that drastically reduces the fluid pressure. Thus, it is currently believed that once the spermatozoa enter the proximal epididymis, their progression is regulated by rhythmic smooth muscle contractions of the epididymal tubule.

It has been demonstrated that the function of the epididymis, and the maturation and survival of spermatozoa within it, depends on hormones secreted by the testis. Spermatozoa develop their fertilizing potential by a complex series of events that occurs in the caput and corpus region of the epididymis, and this process is not intrinsic to the spermatozoa and requires more than passage of time (37-41). Some of the maturational changes, like motility, may be intrinsic to the sperm cell and develops with time, others,
such as the ability to interact with the egg, depend on the epididymal environment (32). The latter has been shown to be conditioned by testicular androgens because sperm maturation occurs after castration, hypophysectomy, or in organ culture only when androgen is administered in vivo or included in the culture medium (42-45). The sequential changes that occur along the epididymis are induced by the epididymal fluid milieu, and the composition of the luminal fluid is controlled regionally by secretions of the epithelium coupled with the selective re-absorption of water, solutes, or suspended material (41, 46, 47). As the maturing spermatozoa move through the different “pools” of epididymal fluid, their surface membrane composition and metabolic processes are altered (48). The surface events that regulate the maturation process are presumed to set off internal transformations that are irreversible, utilize little ATP, and do not require complex biosynthesis. Maintenance and storage of fully mature spermatozoa is primarily the role of the cauda epididymis, and generally, a high percentage of sperm removed from this epididymal region are fertile. In addition to these functional changes, spermatozoa undergo structural changes; the cytoplasmic droplet migrates along the sperm flagellum, the acrosome is reshaped, there are changes in the sperm nuclear chromatin, tail organelles, and in the sperm plasma membrane (32). There is strong evidence, from both in vitro and in vivo studies, that the final stages of the sperm maturation process in all mammalian species studied to date depend on the epididymis (32).
Yoked Hormone-Receptor Model of Constitutive LHR Activation

A transgenic mouse expressing a yoked hormone-receptor (YHR) complex has been developed in order to determine the effect of chronic ligand-mediated LHR activation. The YHR complex was generated by covalently linking the ligand hCG to the rat LHR (figure 3) and is translated as a single polypeptide chain (49). The expression of the YHR complex is under the control of the mouse inhibin α-subunit promoter, which has previously been shown to target transgene expression to the gonads (50). In cell culture, YHR expression has been shown to produce an increase in the basal levels of both cAMP and inositol phosphate as compared to cells expressing LHR (51) and therefore functionally mimics a constitutively active LHR.

Expression of YHR in vivo results in a distinct phenotype. The testis weight is significantly reduced and the seminiferous tubule diameter is significantly smaller in transgenic mice expressing YHR (YHR⁺) when compared to the wild type (WT) animals from 3 weeks onward (figures 4 and 5). YHR⁺ males have increased levels of testosterone at 3 and 5 weeks of age, and seminal vesicle weights of the transgenic animals are greater at 3 and 5 weeks of age due to anabolic effects of testosterone.
Interestingly, after 5 weeks both LH and testosterone levels recover to those of the WT animals, but FSH levels remain lower than those of the WT animals (figure 6) (49). Furthermore, a large number of testes genes were examined using reverse transcriptase polymerase chain reaction and it was found that Leydig cell specific genes encoding enzymes important in testosterone production were significantly lower in YHR\(^+\) animals as compared to WT animals at 3, 5, and 8 weeks of age (52).

![Figure 4. Histological Evaluation of the Testes.](image)

Testes from 5-week-old animals were fixed in Bouin’s and 5mm sections were stained with PAS and hematoxylin. Sections show a decrease in average tubule diameter, as well as smaller Leydig cell cluster size in the YHR\(^+\) animals when compared to the WT controls. Data taken from (1).
Figure 5. Effects of YHR+ Expression on Seminiferous Tubule Development.
Average cross-sectional area of seminiferous tubules in testis from 3 through 12 week-old mice. n=3. Data represent means±S.E.M.; *P<0.05. Data taken from (49).

Figure 6. Serum FSH and Testicular Testosterone Levels in Male YHR+ and WT Mice. FSH (n=4-10) and testicular testosterone (n=6-11) levels were determined by RIA. Testicular testosterone is the total amount per gram testes. Data presented as means±S.E.M.; *P<0.05, **P<0.01, ***P<0.001 compared to WT. Data taken from (49).
Hypothesis

It is hypothesized that the prepubertal elevation in testosterone and persistent decrease in gonadotropin hormones that has been observed in the YHR\(^+\) animals, which mimics the natural mutation that occurs in patients with FMPP, is affecting spermatogenesis or Sertoli cell number/function. This hypothesis will be tested in the following specific objectives:

**Objective 1:** Determine if there is a difference in the germ cell and Sertoli cell populations in WT and YHR\(^+\) animals using flow cytometry and systematic Sertoli cells counts, respectively.

**Objective 2:** Calculate the daily sperm production in the testis and epididymis of WT and YHR\(^+\) animals in order to determine if the decrease in total germ cells calculated in objective one is further decreased by a dysfunction in the epididymis.

**Objective 3:** Determine if the decrease in germ cells observed in YHR\(^+\) animals is the result of decreased proliferation or an increase in either germ cell or Sertoli cell apoptosis. Proliferation will be measured by performing bromodeoxyuridine incorporation assays, while apoptosis will be measured by performing TUNEL assays.
CHAPTER TWO
MATERIALS AND METHODS

Animals and Materials

All the mice that were used throughout the experiments were housed under standardized conditions with a 12-h light cycle. Food (Purina Labdiet 5008) and water were provided to the animals *ad libitum*, and the Institutional Animal Care and Use Committee at Southern Illinois University Carbondale approved all experiments.

Animals were sacrificed at their appropriate time points (±2 days) by carbon dioxide asphyxiation. The weights of the testis and seminal vesicles were recorded as a bioassay for testosterone production, as the testis have an inverse relationship and the seminal vesicles have a direct relationship with testosterone levels. For experiments that did not entail purifying germ cells from the testes, one testis was flash-frozen in liquid nitrogen and stored at -80°C, while the other testis was fixed in Bouin’s solution for use in immunohistochemistry. Fixation in Bouin’s solution was performed by gently poking a hole at each pole of the testis, immersing the tissue in Bouin’s fixative for two hours, and then cutting the testis in half and allowing the tissue to soak in Bouin’s fluid overnight. Testes were then rinsed with six changes of 70% ethanol prior to processing. Blood was collected from animals via a cardiac puncture, and after coagulation at room temperature for one half hour, serum was collected by two cycles of centrifugation at 6000 RPM for 10 minutes, and stored at -80°C.
Table 1. Reagents Utilized

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<th>Use</th>
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<td><strong>Flow Cytometry</strong></td>
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<td></td>
<td>M199</td>
<td>Sigma-Aldrich; St. Louis, MO</td>
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<td></td>
<td>Glucose</td>
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<td></td>
<td>Trypsin</td>
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<td><strong>Sperm Head Resistant Sonication</strong></td>
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<td>Glacial Acetic Acid</td>
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Flow Cytometric Evaluation of Germ Cells

For each flow cytometric analysis, two testes were decapsulated in Medium 199 (M199) that was supplemented with 11.1mM glucose, 1.2mM magnesium sulfate, 6mM sodium L-lactate, 1mM sodium pyruvate, and 25.2mM sodium bicarbonate. Following decapsulation, the testis were placed in an Erlenmeyer flask that contained 20 ml of supplemented M199 with 0.5 mg/ml collagenase and incubated for 20 minutes in a 33°C shaking water bath. The digest was transferred to a 50 ml conical tube, and the seminiferous tubules were allowed to settle to the bottom. After the tubules had settled to the bottom of the conical tube, the supernatant was removed. The seminiferous tubules were washed three times with 10 ml of M199 Medium, followed by incubation in 20 ml of supplemented M199 Medium containing 0.5 mg/ml trypsin and 1 µg/ml DNase for 15 minutes in a 33°C shaking water bath. Seminiferous tubules were manually pipetted for 3 minutes and filtered through a 70 µm cell strainer into a clean 50 ml conical tube. Samples were then centrifuged for 10 minutes at 500 x g. After centrifugation, the pellet was washed twice with 5 ml of supplemented M199 containing 0.5% BSA and 1 µg/ml DNase. The final pellet was resuspended in supplemented M199 Medium. The volume that the final pellet was resuspended in was dependent on the age of the animal: 10 day animals were resuspended in 1 ml, 3, 4, and 5 week old animals were resuspended in 3 ml, and 8, 12, 16, and 24 week old animals were resuspended in 5 ml.

Sertoli Cell Quantification

After Bouin’s fixation, tests were processed and embedded in paraffin. 5µm serial sections were obtained from 5 WT and 5 YHR+ animals at 3, 5 and 8 weeks of age. From
each animal, three sections at least 30 µm apart were used for immunohistochemical staining and Sertoli cell quantification. A goat polyclonal antibody against GATA-4 was used to identify Sertoli cells within the seminiferous tubules.

Slides were dewaxed with Citraclear and dehydrated in ethanol. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in absolute methanol for 30 minutes at room temperature. Slides were blocked with 10% normal donkey serum overnight at 4°C, the slides were then incubated with the GATA-4 primary antibody, diluted 1:400, for one hour at room temperature. Sections were then incubated with a horse anti-goat biotinylated secondary antibody for 10 minutes at room temperature, followed by Vectastain® Elite ABC reagent for 10 minutes at room temperature. GATA-4 expression was visualized using diaminobenzidine (DAB), which produces brown deposits. Lastly, the sections were counterstained with hematoxylin, dehydrated and mounted.

Sertoli cell quantification was done without prior knowledge of the genotype. After immunohistochemistry, twenty tubules that were equally distributed across the section and round in shape were randomly selected to be counted. From each animal, a total of 60 tubles were counted. The Sertoli cell populations were analyzed by taking the average number of Sertoli cells per tubule and the relative percent Sertoli cells in YHR+ compared to WT animals.

**Bromodeoxyuridine (BrdU) Incorporation Assays**

5 WT and 5 YHR+ animals at 3, 5, and 8 weeks of age were used for BrdU incorporation assays in order to examine the proliferation of germ cells. Animals were
given a single intraperitoneal injection of BrdU (in 0.9% sterile saline) at a dose of 150mg/kg body weight and were sacrificed two hours later. Testes from these animals were removed, weighed, and fixed in Bouin’s solution. Following fixation, the testes were embedded in paraffin wax and cut into 5µm thick sections. For each animal, 3 sections at least 30 µm apart were used for quantification purposes. The slides used had been prepared and made available for staining through the work of a previous graduate student in the lab (1). In order to stain for proliferating germ cells, a mouse monoclonal antibody against BrdU was utilized.

Sectioned slides were dewaxed with CitraClear and dehydrated in ethanol. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in cold absolute methanol for 30 minutes. Slides were blocked with mouse-on-mouse (M.O.M.) block, prepared according to the manufactures instructions, for 3 hours at 4°C. Sections were then treated with M.O.M. diluent, prepared according to the manufactures instructions, for 5 minutes, and then incubated with the anti-BrdU primary antibody, diluted 1:1000, for 1 hour at room temperature. Following incubation with the primary, slides were incubated with the M.O.M. biotinylated anti-mouse secondary antibody for 10 minutes at room temperature, followed by Vectastain® Elite ABC reagent for 10 minutes at room temperature. BrdU incorporation was visualized using DAB.

Three hundred round seminiferous tubules were randomly selected from each animal to be counted without prior knowledge of their genotype. The percent of BrdU positive tubules, and the number of BrdU positive germ cells in each seminiferous tubule were quantified.
Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assays

Three sections from at least 3 WT and 3 YHR\(^+\) animals at 10 days, 2, 3, 5, and 8 weeks of age were utilized for TUNEL assays to examine the level of germ cell apoptosis. Slides and quantification of apoptotic germ cells was made available for use through previous experimentation.

Slides were dewaxed in Citraclear and dehydrated in ethanol. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in absolute methanol. Sections were permeabilized with Proteinase K for 30 minutes at 37°C. Slides were incubated with TUNEL enzyme, diluted 1:25 in TUNEL labeling mix and dilution buffer. Sections were blocked in 5% normal donkey serum for 10 minutes at room temperature and then incubated for 30 minutes with hors-radish peroxidase conjugated sheep anti-fluorescein antibody at 37°C, which allows analysis using a light microscope. Apoptotic cells were visualized with DAB. Slides were counterstained with hemotoxylin, dehydrated in isopropanol, and mounted.

The entirety of each section was analyzed and the number of apoptotic germ cells per tubule was quantified. The average number of apoptotic cells per tubule was calculated by counting the total number of apoptotic cells in each section and dividing this by the number of tubules with apoptotic germ cells.

Sonication Resistant Sperm Heads

In order to compare daily sperm production between WT and transgenic animals, testes, epididymides, and seminal vesicles from 7 WT and 8 YHR\(^+\) males of 12 weeks of
age were removed and weighed. The seminal vesicles were weighed as a bioassay for testosterone production. The tunica albuginea was removed from one testis and the seminiferous tubules were homogenized at a medium speed in 1 ml of 0.15M NaCl and 0.05% triton X-100 for 10 seconds. One epididymis from each animal was divided into its three primary regions: the caput, corpus, and cauda. The caput was minced gently with fine dissecting scissors prior to being combined with the corpus region, while the cauda region was prepared by itself. Following homogenization of the testis, caput/corpus, and cauda, samples were sonicated at medium power for 3 minutes at 20 second intervals on ice. After sonication, the testis sample was diluted in 2 ml of the homogenization buffer and filtered through a Whatman grade number 2 filter. Samples were either counted immediately or stored at 4°C for no more than 24 hours. Spermatozoa nuclei were counted using a hemocytometer under a microscope that was fitted with a 40x objective and phase contrast optics. A counting field was selected such that at least 200 spermatid heads were counted. Generally, this was achieved by counting the center 25 boxes (1/25 sq. mm) so that the total volume was equivalent to 0.1µl, and then the total number of sperm heads per milliliter were calculated by dividing the number of sperm heads counted by 0.1 and multiplying by the total volume that the sample was suspended in.

2D Electrophoresis and Image Analysis

Protein extracts were prepared by mincing the respective epididymal regions in 1 ml of M2 medium, incubating for 15 minutes at 37 °C to activate and release the sperm. The sperm was transferred to tubes and cell debris was allowed to settle by gravity. The supernatant containing the sperm was layered on a 1 ml cushion of 25% Percoll that was
prepared in M199 media and centrifuged at 250 x g for 20 minutes, in order to remove sperm debris. The sperm pellet was washed twice and centrifuged for 10 minutes at 13 x g. Protein pellets were stored at -80 °C until further usage.

The protein extract was prepared for 2D electrophoresis by resuspending the necessary protein pellets in 500 µl of 7M Urea, 2M thiourea, 4% CHAPS, 50 mM DTT, and 0.5% Sigma protease inhibitor cocktail. Samples were then sonicated with 3 to 4 short bursts at setting 6, and vortexed for about 10 seconds every 10 minutes for one hour. Samples were then centrifuged for 30 minutes at 13000 x rpm, and supernatant was transferred to a new Eppendorf tube. The concentration of the protein extract was calculated using the BioRad Protein Assay kit that is based on the Bradford method. Corpus sperm samples were diluted 1:5 and 1:10 while cauda sperm samples were diluted 1:10 and 1:20 in water, for a final volume of 30 µl. Diluted sperm protein samples were read in duplicates using a BD Falcon 96 well flat bottom flexible plate. 200 µl of Bio-Rad Protein Assay dye, diluted 1:5, was added to each well of diluted protein and read at 562nm using a Biotek Synergy 2 multi-detection microplate reader.

Total protein extract (150 µg) from the caput, corpus, and cauda spermatozoa was used for the 2D electrophoretic analysis. Sample was initially hydrated overnight on a 11 cm BioRad IPG gel strip with a 3-10 nonlinear pH gradient. The next day IEF (iso-electric focusing) was performed with the Protean IEF Cell (BioRad, Hercules, CA). Equilibration of the strips was according to the manufacture’s instruction. Linear SDS PAGE gradient gels (8% to 16% w/v) were used for the resolution of proteins in the second dimension. A BioRad Criterion Cell apparatus was used for gel electrophoresis at 100 volts for 90 to 100 minutes at ambient temperature (25±2 °C). Gels were washed
with distilled water and stained for 5 minutes with 0.5% Coomassie Brilliant Blue G-250 that was prepared in 50% HPLC grade methanol/ 10% acetic acid. Gels were briefly rinsed with MilliQ water to remove any residual stain and then destained with 40% HPLC grade methanol/ 10% acetic acid. The destaining solution was changed every 45 minutes until faint protein spots are identifiable, followed by destaining of the background overnight in MilliQ water.

2D Electrophoresis Image Analysis

Acquisition of gel images used a high-resolution laser scanner (Odyssey infrared imaging system from LI-COR, Lincoln, NE) that can be used to detect coomassie stained gels. Images were analyzed with BioRad PDQuest Basic software (BioRad). The software identifies spots and quantifies pixel intensity within the spot boundary. The initial analysis is automated, followed by a manual edit to ensure that each spot was matched appropriately with the other gels within the replicate group. The replicate group tool allows replicate gels to be viewed together and spot identity can be manually verified. The volume for each protein spot was normalized against the total spot intensity, and the software performs Students T-test (p <0.05) to determine whether a spot undergoes a statistically significant change in intensity.

Statistical Analysis

Data are expressed as mean ± S.E.M.. Data were analyzed by nonparametric Students T-test or by 2-way ANOVA with Bonferroni’s post-test using the Prism software program (GraphPad Software, Inc.; San Diego, CA).
CHAPTER THREE
GERM CELL AND SERTOLI CELL QUANTIFICATION

**Objective 1:** Quantify the difference in the germ cell and Sertoli cell populations in WT and YHR\(^+\) animals using flow cytometry and systematic cell counts, respectively.

**Objective 1 Rationale**

It has previously been reported that the testes weight of YHR\(^+\) animals is significantly decreased from 3 weeks of age onward, when compared to age matched WT controls (49). It has also been observed that the seminiferous tubule diameters of YHR\(^+\) males are smaller than those of equal aged WT controls from 3 weeks onward (figure 5). Taken together, these observations suggest that the decrease in seminiferous tubule diameter may result from YHR\(^+\) males being able to support fewer germ cells, which ultimately may be caused by the presence of fewer Sertoli cells, since Sertoli cells are capable of supporting a finite number of germ cells. To support this, FSH has been shown to support the proliferation of Sertoli cells during the neonatal period, which determines the final Sertoli cell population and overall testis size. Because the Sertoli cell is the only cell that contains the FSH receptor, it is believed to be the key modulator of spermatogenesis (53).

**Body and Testis Weight**

The body weights of WT and YHR\(^+\) animals follow relatively the same trend from 10 days through 24 weeks (figure 7, left). Interestingly, at 4 weeks of age YHR\(^+\)
animals weighed significantly less than their WT counterparts, but at 8 weeks of age YHR\(^+\) animals weighed significantly more than WT animals. For testis weight, YHR\(^+\) animals exhibited a significant decrease in testis size at 3 through 16 weeks of age (figure 7, right). The decrease in weight is most severe at 6 weeks of age (54% reduced, n=10), but is apparent by 3 weeks of age (35% reduced, n=18). At later ages, the difference in testicular size is less pronounced, however, but YHR\(^+\) weights are still significantly reduced at 8 weeks (34% reduced, n=18-20), 12 weeks of age (12% reduced, n=15), and 16 weeks of age (20% reduced, n=12). At 24 weeks of age YHR\(^+\) testis weighed less than their WT counterpart but did not reach statistical significance (10% reduction, n=7-10).

**Germ Cell Quantification**

Differences in total germ cells and the individual germ cell populations were quantified using flow cytometry. This method has been shown to be effective at analyzing complex populations of cells based on molecular markers, or in this case, changes in ploidy levels. Based on the degree of fluorophore binding, different histogram peaks are created due to the different quantity of DNA that each germ cell population contains. For example, spermatids have one copy of DNA so they have a certain fluorescent point. Spermatogonia have two copies of DNA so their fluorescence intensity will be twice that of the haploid spermatids, and the same trend is true for the spermatocytes, which have four copies of DNA (figure 8).

A comprehensive analysis of the temporal changes in germ cell populations was done on neonatal, prepubertal, and adult animals as a qualitative marker for spermatogenesis. Relative quantities of each population were quantified by calculating
the percent of cells under each histogram peak relative to the total number of germ cells calculated for each animal. Calculating the relative number of cells under each histogram peak allows for comparison between genotypes, as well as with different ages. The results for this experiment are shown in figure 9.

Beginning at 10 days of age, there is a significant decrease in the total number of germ cells in YHR\(^+\) animals relative to WT controls (figure 9). Beginning prepubertally, after 3 weeks of age the difference in the total cells per animal becomes evident as the YHR\(^+\) have significantly fewer cell from 4 weeks through 24 weeks. Interestingly, at 5 weeks of age there is a spike in the total number of germ cells, and in all the individual populations, for the WT animals, but this trend is not seen in the YHR\(^+\) animals.

The diploid cell population consists of spermatogonia cells, which are the precursor cells from which all other germ cell populations develop, secondary spermatocytes, which represent the cells that can be found during the short duration of time between meiosis I and II, and somatic cells. However, because the secondary spermatocytes are short lived, and the somatic cells represent a very small percent of the total testicular cells, the majority of cells in the diploid population are spermatogonia. From 10 days to 3 weeks of age there is a rapid increase in the number of diploid cells in both the WT and YHR\(^+\) animals, but from 3 weeks to 24 weeks the YHR\(^+\) population remains relatively stable while the WT population spikes at 5 weeks and slowly increases through 24 weeks (figure 9). Quantification of the spermatogonia and the secondary spermatocytes revealed that there was a significant decrease at 10 days and then again from 4 weeks to 24 weeks there were significantly fewer diploid cells in the YHR\(^+\) animals.
Primary spermatocytes comprise the tetraploid population. The proliferation of type B spermatogonia results in production of primary spermatocytes that enter and undergo the first meiotic division, resulting in primary spermatocytes. This cell population showed a very similar trend to that seen for the spermatogonia and secondary spermatocytes. From 10 days to 3 weeks there was a rapid increase in the population of both WT and YHR\(^+\) animals, and then from 3 weeks through 24 weeks the YHR\(^+\) population remained relatively stable (figure 9). However, at 5 weeks the WT animals had the most dramatic population spike as compared to any other individual population, and then from 8 weeks through 24 weeks the WT trend nicely mirrored the YHR\(^+\). When quantified, at 10 days and from 4 weeks to 16 weeks of age there are significantly fewer primary spermatocytes in the YHR\(^+\) animals compared to their WT counterparts. A decrease in spermatocyte number was observed in the 3 week and 24 week YHR\(^+\) compared to WT mice; however it did not reach significance.

Lastly, the spermatids represent the final population of cells that are produced during spermatogenesis. Because of the physical nature of the purification procedure that is required for quantifying germ cells, round spermatids were primarily quantified, and elongated spermatids were lost during the wash steps. The spermatid population begins to be quantifiable at 3 weeks, and steadily increases in number through 24 weeks (figure 9). In the WT animals the spermatid population dramatically increases until 5 weeks of age and the slowly increases through 24 weeks. In the YHR\(^+\) animals, there is not a dramatic increase up to 5 weeks, rather the spermatid population steadily increases through 24 weeks. The relative spermatid population is significantly decreased in YHR\(^+\) animals at 5, 8, and 12 weeks, as compared to the WT. Taken together, these data indicate that there is
a decrease in the spermatogonial population that could be resulting in a decrease in all subsequent populations.

Figure 7. Average Body Weights and Testes Weights of WT and YHR⁺ Mice. Body weights (left) in animals from 10 days to 24 weeks of age. n=7-10. Testis weights (right) in animals from 10 days to 24 weeks of age. n=7-52. Data represent mean±S.E.M. *p<0.05, ***p<0.001 compared to WT.
Figure 8. Flow Cytometric Analysis of Total Germ Cells from 3 and 5 Week Old WT and YHR\textsuperscript{+} Animals. The histogram represents the frequency of haploid (1n), diploid (2n), and tetraploid (4n) germ cells from 3 week (A, C) and 5 week (B, D) animals. Note the change in the distribution of germ cell populations in both WT (A, B) and YHR\textsuperscript{+} (C, D) between the two ages. From this type of analysis, the ratios of the different germ cell populations were quantified between genotypes and during development.
Figure 9. Quantification of Total and Developing populations of Germ Cells in WT and YHR<sup>+</sup> Mice From 10 Days to 24 Weeks of Age. Germ cell data from WT and YHR<sup>+</sup> animals was determined using flow cytometry. The data represent mean±S.E.M. (n = 8-10). Significant differences between genotypes are indicated by * p<0.05 **p<0.01 ***p<0.001.
Sertoli Cell Quantification

Precursor Sertoli cells develop from undifferentiated mesenchymal cells located in the gonadal ridge. Sertoli cells begin to proliferate in the fetus and cease around 15-20 days postnatal in the mouse. Because the adult Sertoli cell population is established by 3 weeks of age, this time point was chosen to be the starting point for quantification.

For the purpose of this study, Sertoli cells were quantified based on specific staining with GATA-4 and morphological characteristics. Representative sections illustrating the specificity of Sertoli cell staining and morphology can be seen in figure 10. Mature Sertoli cells have a tripartite nucleus, stain brown, and are located towards the basement membrane in sexually mature animals. Three hundred round seminiferous tubules were randomly selected from each animal to be counted without prior knowledge of their genotype. The percent of BrdU positive tubules, and the number of BrdU positive germ cells in each seminiferous tubule were quantified.

Figure 10. Representative Section of a 5 Week Old GATA-4 Stained Testes. Within the seminiferous tubules, GATA-4 transcription factor stains Sertoli cells but not germ cells. Arrows indicate positively stained Sertoli cells in WT (left) and YHR⁺ (right) animals.
Congruent with the cessation of Sertoli cell proliferation, after 3 weeks of age there was no further increase in the number of Sertoli cells per tubule (figure 11, left). At all ages measured, there was no significant difference observed in the number of Sertoli cells per tubule or percent Sertoli cells in YHR\(^+\) animals compared to WT (figure 11). Interestingly, at both 5 and 8 weeks of age, there were more Sertoli cells per tubule in YHR\(^+\) animals as compared to WT animals; 18% and 14% increase respectively (figure 11, right).

When these data are taken together with the quantification of the total number of germ cells it is demonstrated that the decrease in YHR\(^+\) germ cell number at 10 days and from 4 weeks through adulthood is independent of the Sertoli cell population.

![Figure 11](image)

**Figure 11. Quantification of Sertoli Cells From 3, 5, and 8 Week Old Animals.**
Number of Sertoli cells per tubule and relative percent of Sertoli cells in YHR\(^+\) animals compared to their WT counterparts. Data are expressed as mean±S.E.M. (n= 3-5).
CHAPTER FOUR

DAILY SPERM PRODUCTION MEASURED IN THE TESTIS AND EPIDIDYMIS

Objective 2. Determine the daily sperm production in the testis and epididymides of animals.

Objective 2 Rationale

It has previously been reported that testosterone and FSH act as a germ cell survival factor during the initiation of spermatogenesis (53). However, the precise relationship between testosterone and FSH in the hormonal regulation of spermatogenesis has not been fully elucidated. In addition to this, it is not currently known how premature elevations of testosterone in combination with a persistently low level of FSH affect daily sperm production. In our lab, it has previously been shown that YHR\(^+\) mice are sub-fertile compared to WT mice. A preliminary breeding study was conducted for six months, in which, male YHR\(^+\) and WT littermates were mated continuously with WT female mice. The results indicate that, although there was no difference in the average size of the litters, YHR\(^+\) mice sired significantly fewer litters and the average days between litters was greater for YHR\(^+\) males (data not shown). In order to determine if YHR\(^+\) animals are producing less sperm, sonication resistant spermatozoa nuclei from adult animals were quantified from the testis and epididymides. This analysis will also reinforce the haploid germ cell counts performed using flow cytometry. The protocol that is used to isolate germ cells for flow cytometry results in an inherent variability in the number of elongated spermatids collected, and therefore, this procedure is a good way to look at the elongated spermatids in the testis and epididymis. Counting of spermatozoa nuclei is made possible because as the spermatid nucleus elongates structural changes
occur to the chromatin which allows the nucleus to become resistant to sonication beginning at step twelve in the mouse spermatogenic cycle (54).

Spermatozoa Nuclei Quantification

Representative pictures of sperm heads in a hemocytometer grid that was used for quantification can be seen in figure 12, while the quantification of the spermatozoa nuclei can be seen in figure 13. No significant difference was observed in the daily testicular sperm production or in the sperm reserves of the cauda epididymis at either 12 or 16 weeks of age, although there is a 12% decrease in caudal sperm reserves at 12 weeks for YHR\(^+\) animal. This decrease seen at 12 weeks was offset by a 4% increase at 16 weeks in caudal sperm reserves. Significantly fewer spermatozoa nuclei were found at 12 weeks of age in the pooled caput/corpus region of the YHR\(^+\) epididymis when compared to WT animals at the same age. However, at 16 weeks of age there was only a 14% decrease in pooled caput/corpus spermatozoa as compared to the 36% decrease seen at 12 weeks of age. Consistent with work that was conducted by Glover and Nicander (55), the caput and corpus region have fewer spermatozoa nuclei than the cauda region because the proximal segments of the epididymis are specialized for sperm maturation, while the distal segment is specialized for sperm storage. The caput region of the epididymis was pooled with the corpus region for this experiment because testicular fluid, and therefore, spermatozoa progress rapidly through the initial segment of the caput and begins to slow down by the distal segment (32). This rapid transit through part of the caput can lead to variability in the quantity of spermatozoa that are present in that region.
Although there were significantly fewer spermatozoa nuclei in the caput/corpus region of the YHR\(^+\) animal at 12 weeks of age no difference was observed for any other region tested. These data indicate that the elongated spermatid population and spermatozoa nuclei are comparable in the WT and YHR\(^+\) animals at 12 and 16 weeks of age.

Figure 12. Representative Image of Sonication Resistant Spermatozoa Nuclei From an Adult Testis.
Figure 13. Daily Sperm Production From the Testis and Epididymis of WT and YHR⁺ Mice. One testis and one epididymal region were used for quantification of 12 week (panel A) and 16 week old mice (panel B). **p<0.01  The data represent mean±S.E.M. (n=4-8).
CHAPTER FIVE

EVALUATION OF PROLIFERATION AND APOPTOSIS IN GERM CELLS

Objective 3: Determine if the decrease in YHR+ germ cell number is due to a decrease in proliferation or an increase in apoptosis.

Objective 3 Rationale

The role of testicular androgens, primarily testosterone and dihydrotestosterone, in initiation, maintenance, and restoration of spermatogenesis has been an area of research for decades (53, 56). However, the details of the cellular and molecular mechanisms of androgen action within the testis still remain ill defined. FSH, on the other hand, has an indirect action on germ cells via the Sertoli cell. FSH is the primary endocrine hormone regulating Sertoli cell function and proliferation, and the mechanism by which FSH exerts its effect on the spermatogonia is believed to be as a survival factor rather than a proliferative factor (53). Three weeks of age was selected to be the starting point for this experiment because in the mouse Sertoli cells will divide and differentiate until 15-20 days of age (22). From 21 days onward the Sertoli cell population has been established, and thus, able to support a full complement of germ cells. Germ cell proliferation was examined using bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA strands. Immunohistochemical staining with anti-BrdU antibody allowed for the quantification of proliferating spermatogonia based on morphology and location within the seminiferous tubules.

In an effort to verify that the decrease in germ cell numbers is not a consequence of an increase in apoptosis, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) was performed and evaluated. There is no evidence that elevated
levels of testosterone induce apoptosis, low levels of FSH could be affecting the survival rate of spermatogonia. This assay was utilized to ensure that the differences in total germ cell number, as well as with in the germ cell sub-populations, is not a result of an increase in cell death.

**Germ Cell Proliferation**

BrdU is a thymidine analog that selectively incorporates into cells during the S-phase of the cell cycle. For this reason, BrdU can be utilized as a marker for active cell proliferation. Representative BrdU immunohistochemistry sections are shown in figure 14 and quantification of proliferating germ cells are shown in figure 15.

Spermatogonia, which include type A and type B forms, are the most immature germ cells. The actual stem cell pool for the germ cell line is considered to be a subset of type A spermatogonia, the A pale spermatogonia, while type B spermatogonia are considered to be primary spermatocytes once they enter meiosis I and give rise to secondary spermatocytes. In all mammals, both type A and B spermatogonia undergo a series of mitotic divisions to produce a large number of germ cells that are available for entry into meiosis. Based on this, the size of the spermatogonial population is a key determinant in the eventual number of mature sperm produced. In the WT animal the peak of proliferation is at 5 weeks of age and by 8 weeks the proliferation levels had returned to those measured at 3 weeks. In the YHR^+^ animals, there is no distinct peak in proliferation, and the levels remain relatively constant from 3 weeks to 8 weeks. It is observed that at 5 weeks of age there was a 17% reduction in the number of proliferating cells in the YHR^+^ animals as compared to the WT controls.
Figure 14. Representative Sections of 3 Week-Old WT and YHR\(^+\) BrdU Stained Testis. Testes were immunohistochemically stained with an antibody against BrdU. BrdU expression was visualized using a peroxidase detection system, with DAB serving as the substrate, causing the nucleus of BrdU positive cells to stain brown.

Figure 15. Germ Cell Proliferation. Quantitative evaluation of the percent BrdU positive tubules and the total number of BrdU positive cells was performed by randomly selecting 300 round tubules per animal and determining the number of germ cells that were positively stained. Results are expressed as mean ± SEM (n=3-5). Data was analyzed using an unpaired students t-test. P-value < 0.05.
**Germ Cell Apoptosis**

Representative TUNEL sections are shown in figure 16. Sections from at least three animals of each genotype at 10 days, 2, 3, 5, and 8 weeks of age were used for this study. As seen in figure 17, there is no significant difference in the number of apoptotic cell per tubule at any time point analyzed. It is worth noting that there were 30% fewer apoptotic cells per tubule in YHR\(^+\) animals at 10 days and 5 weeks of age (figure 17, panel A). Also, in panel B, the YHR\(^+\) animals had relatively the same number of apoptotic cells per tubule over time, while in the WT controls there was more fluctuation across the time points.

When comparing the percent of tubules with apoptotic cells, both the WT and YHR\(^+\) animals showed the same trend. For both genotypes, the greatest number of apoptotic tubules was recorded at 3 weeks of age, with a steady decline occurring at 5 and 8 weeks respectively (figure 18, panel A). Also, at 3 weeks of age there were 31% more tubules with apoptotic cells in the YHR\(^+\) animals than the WT controls (figure 18, panel A).

Together, the proliferation and apoptosis data indicate that the significantly lower level of FSH in the YHR\(^+\) animals do not significantly affect the rate of germ cell proliferation or apoptosis.
Figure 16. Representative TUNEL Sections of 3 Week-Old WT and YHR<sup>+</sup> Animals. The 3’ end of the cleaved DNA was enzymatically labeled using TdT enzyme and apoptotic cells were visualized with DAB. Arrows indicate apoptotic germ cells.
Figure 17. Number of Apoptotic Cells per Tubule. Quantitative analysis of apoptotic germ cells was performed using a TUNEL assay. The number of seminiferous tubules that had positive TUNEL staining was counted followed by the number of positive TUNEL cells within those tubules. Results expressed as mean ± SEM (n=3-5). Data was analyzed using an unpaired students t-test. P-value < 0.05.
Figure 18. Percent of Seminiferous Tubules with Apoptotic Cells. All seminiferous tubules were counted in each section followed by the number of tubules that have apoptotic cells. Results expressed as mean ± SEM (n=3-5). Data was analyzed using an unpaired students t-test. P-value < 0.05.
CHAPTER SIX

EVALUATION OF EPIDIDYMAL SPERMATOZOA PROTEIN USING TWO-DIMENSIONAL GEL ELECTROPHORESIS

Objective: Determine if early increase in testosterone, due to chronic activation of LHR, changes the protein composition of sperm during transit through the epididymis and if there are differences in the WT and YHR\textsuperscript{+} proteomic profiles.

Rationale

Immature sperm that leave the testis lack the necessary properties essential for fertilization, such as, forward motility and the ability to bind and penetrate to the zona pellucida. These properties are acquired during the transit of the spermatozoa through the caput and corpus epididymis. The functional competence that is gained with epididymal transit is paralleled by the acquisition of flagellar beating (motility) and compacting of the nuclear and flagellar structures (morphological).

The acquisition of fertilizing ability during epididymal transit is what defines the concept of sperm maturation. The intriguing facet of this maturation is that functional competence is acquired in the absence of transcription and translation, because transcriptional activity is terminated or limited in late elongating spermatids (33). Epididymal protein synthesis and secretion are regulated by testicular androgen, and the pattern of gene expression along the epididymis varies from one segment to another. The maturational process of spermatozoa depends on the sequential modification of the sperm surface resulting from interactions with different interluminal fluids that have different macromolecule compositions. This causes the spermatozoa plasma membrane to undergo changes in the protein composition and localization. Carbohydrate moieties, such as
glycosylphosphatidylinositol (GPI)-anchored integral membrane proteins or oligosaccharide moieties, on the surface of the spermatozoa that face the luminal fluid are subject to modification by enzymes secreted from the luminal epithelium. A deficiency in these enzymes is implicated in male infertility (57). Much of the work that has been conducted to date focuses on an androgen deprived state, rather than early and persistent production. There have also been reports that suggest LH can directly regulate epididymal function (58). These observations are supported by the presence of LHR transcripts and protein in the epididymis, and LH action has been shown to be important for plasminogen activator-mediated sperm maturation in monkeys (59, 60).

It has previously been shown in a preliminary breeding study that male transgenic mice expressing YHR were subfertile, have fewer testicular germ cells, but have the same caudal sperm reserve. Based on this, the goal of this study was to determine changes in protein composition during epididymal transit, and since the YHR+ mice are sub-fertile, any differences in the protein composition of epididymal sperm of YHR+ mice would be able to be identified using two-dimensional electrophoresis.

2D Electrophoresis and Image Analysis

In each experiment, protein samples from each epididymal region were analyzed on three replicate gels. Representative gels of sperm protein from the corpus and cauda regions of WT and YHR+ mice are shown in figure 19. Gels from four independent experiments were analyzed using the PDQuest 8.0 software and comparisons were made between genotypes for each epididymal region: WT caput v. YHR+ caput, WT corpus v. YHR+ corpus, WT cauda v. YHR+ cauda. Also analyzed were proteomic changes that
occur during the transit of sperm along the epididymis within a genotype (i.e. caput v. corpus, corpus v. cauda, and caput v. cauda). The analysis parameters were set to detect, at a minimum, statistically significant two-fold changes in spot intensity, with 95% confidence limits. In these experiments, we detected on average between 300 and 500 spots from each region, with the caput region being on the low end and the cauda region being on the upper end of the averages. The caput region was excluded from analysis due to low protein abundance and high levels of variability in the proteomic expression, and overall lack of consistency.

Differentially abundant proteins were identified from four independent experiments that were analyzed separately. Spot matches were determined manually based on its location on the gel. In total 276 gels were run and 48 were used in the final quantification of differentially abundant proteins. For all experiments, the correlation coefficient was 75% or better. The comparison analysis used 3 technical replicates from 7 and 15 pooled animals for the cauda and corpus samples respectively. The pI for the majority of the protein spots ranged from a pH of 3 through 10, and the molecular weights were between 95 and 17 kDa. When comparing differentially abundant proteins between genotypes 3 corpus protein spots were consistently different in all four experiments, and zero cauda proteins were consistently different between all four experiments. One protein spot was differentially expressed in the YHR\(^+\) sperm as it transited from the corpus to the cauda in all four experiments whereas no changes were detected in WT sperm during transit. The results from the four experiments are summarized in table 2.
Figure 19. 2D Electrophoresis Images Obtained from the Corpus and Cauda Epididymis of YHR\(^+\) and WT Adult males. The purple box represents a corpus protein spot that was differentially expressed between genotypes for all four experiments. Orange box represents a cauda protein spot that was differentially expressed between genotypes for all four experiments. Red box represents a protein spot that was differentially expressed in the WT animal as it transited from the corpus to the cauda in three of four experiments. Blue box represents a protein spot that was differentially expressed in the YHR\(^+\) animal as it transited from the corpus to the cauda in all four experiments.
Table 2. Summary of 2D Electrophoresis Experiments.
The comparison analysis used 3 technical replicates from 7 and 15 pooled animals for the cauda and corpus samples respectively, and 4 independent experiments were performed.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total Matched to all Gel Members of Experiment</th>
<th>Corpus Spots (Average)</th>
<th>Corpus Spots (Average)</th>
<th>Spots Matched Across 4 Experiments (between Genotypes)</th>
<th>Spots Matched Across 3 Experiments (Transit)</th>
<th>Spots Matched Across 3 Experiments (Transit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 18</td>
<td>181</td>
<td>WT Avg. = 531 YHR' Avg. = 510</td>
<td>WT Avg. = 385 YHR' Avg. = 440</td>
<td>Corpus = 3 Cauda = 0</td>
<td>WT = 0 YHR' = 1</td>
<td>WT = 2 YHR' = 1</td>
</tr>
<tr>
<td>Experiment 19</td>
<td>150</td>
<td>WT Avg. = 397 YHR' Avg. = 403</td>
<td>WT Avg. = 439 YHR' Avg. = 386</td>
<td>Corpus = 5 Cauda = 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 21</td>
<td>132</td>
<td>WT Avg. = 303 YHR' Avg. = 376</td>
<td>WT Avg. = 379 YHR' Avg. = 353</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 22</td>
<td>134</td>
<td>WT Avg. = 438 YHR' Avg. = 394</td>
<td>WT Avg. = 417 YHR' Avg. = 430</td>
<td></td>
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</table>
CHAPTER SEVEN

DISCUSSION, CONCLUSION, AND FUTURE DIRECTIONS

Spermatogenesis is a series of complex biochemical events that is dependent upon the proper regulation of the hypothalamic–pituitary–testicular axis. Hormones such as testosterone, FSH, and LH are known to influence germ cell fate, in that, removal will induce germ cell apoptosis. More specifically, the action of testosterone is absolutely necessary for germ cells to progress through meiosis, while FSH primarily serves to promote spermatogenic output by increasing the number of Sertoli cells. Together, testosterone and FSH have an additive effect on the entry of germ cells into meiosis but act synergistically to stimulate completion of meiosis and the entry of the germ cell into spermiogenesis (26).

In the YHR mouse model testicular testosterone levels are significantly elevated beginning at 10 days of age and remain significantly elevated at 2 weeks and 5 weeks but not at 3 weeks of age. In contrast, serum FSH is significantly decreased in YHR+ mice beginning at 2 weeks of age and remains significantly decreased through adulthood. As a result of alterations in circulating hormone levels, spermatogenesis in YHR+ mice is altered, which results in quantitatively fewer germ cells. As more data are being acquired, it appears that the Sertoli cells in YHR+ mice are supporting a smaller population of germ cells, leading to these mice having smaller testis and a smaller seminiferous tubule diameter.

Although the direct action of testosterone and FSH in regulating spermatogenesis remains elusive, more evidence is mounting that supports the synergistic behavior of these molecules in working towards the common goal of germ cell survival.
Contrary to what was predicted, there was no significant difference calculated for either germ cell proliferation or apoptosis in the YHR\(^+\) mice as compared to their WT counterparts even though serum FSH levels are significantly decreased beginning at 2 weeks of age. This indicates that by puberty the recovery of testosterone levels is compensating for low FSH levels in YHR\(^+\) males. These data, in combination with the Sertoli cell counts, indicates that YHR\(^-\) animals have comparable Sertoli cell numbers to the WT animals and comparable levels of germ cell proliferation and apoptosis. The difference between the genotypes seems to reside in the germ cell to Sertoli cell ratio.

Thorough evaluation of testicular germ cells using flow cytometry indicates that YHR\(^+\) animals produce significantly fewer germ cells at 4 weeks of age and remain significantly decreased through adulthood. Corresponding to this, there were significantly fewer spermatogonia detected beginning at 4 weeks of age, and because spermatogonia are the precursor cell from which all other germ cells develop from, there are significantly fewer spermatocytes and spermatids beginning at this age. There was a degree of variability in the spermatid population, which is potentially due to the difficulty in recovering the elongated spermatid population during the purification procedure. Quantification of sonication resistant sperm heads in adult animals in order to determine the elongated spermatid population showed a decrease but it was not significant.

These data further support what is already known about FSH and its role in maintaining Sertoli cell Function. First, FSH has been hypothesized to play a role in determining the number of stem cell niches for A type spermatogonia (53). More specifically, stem cell renewal is positively regulated by glial cell line-derived neurotrophic factor (GDNF), and decrease in FSH levels will result in low GDNF levels
in the testis. Second, FSH and testosterone are believed to interact at the level of communication between spermatids and Sertoli cells. This was shown using co-culture experiments, in which immature rat Sertoli cells were incubated with round spermatids, and only when testosterone and FSH were administered concomitantly was spermatid density doubled (61). It has also been shown that FSH but not testosterone alters the distribution of Sertoli cell-spermatid junction related cytoskeletal proteins, and was concluded that FSH induces the spermatid-binding competency, whereas testosterone acts to enhance the binding of spermatids to the Sertoli cell.

The conundrum with the overlapping properties of FSH and testosterone is that they have the ability to compensate for one another, making it difficult to isolate the precise function of each hormone. It is possible that the significant decrease in serum FSH concentration beginning at 2 weeks of age in YHR\(^+\) mice is being partially masked by the normal serum testosterone concentration in adult animals, which is making the effects of low FSH more elusive to determine. It is possible that low serum concentration of FSH could be affecting the early qualitative development of Sertoli cells without affecting them quantitatively. For example, no difference was observed in the number of Sertoli cells between the WT and YHR\(^+\) animals, but there are significantly fewer germ cells in the YHR\(^+\) animals beginning at 4 weeks of age. This seemingly inconsistent finding may be explained by the synergistic overlap between testosterone and FSH. The serum concentration of FSH is significantly decreased in YHR\(^+\) animals beginning at 2 weeks of age, and serum concentration of testosterone remains significantly increased in YHR\(^+\) until 5 weeks of age. It is possible then that the YHR\(^+\) animal is able to establish the same number Sertoli cells in the brief 15-20 day window of proliferation after birth.
The quantitative decrease in germ cell production can be correlated to the essential need for FSH within this brief window of proliferation for the formation of tight junctional complexes between adjacent Sertoli cells and for the initiation of the first wave of spermatogenesis (62, 63). In the prepubertal animal, FSH influences most of the synthetic and metabolic processes of the Sertoli cell. This includes differentially stimulating Sertoli cells to produce androgen binding protein as well as a number of mitogens and growth factors. Arguably, the most important role of FSH is supporting the successful structural maturation of the Sertoli cell and the increase in protein secretion in the lumen of the seminiferous tubules that allows for successful spermatogenesis (62).

In order to further investigate the decrease in total testicular germ cells observed in YHR\(^+\) males, daily testicular sperm production counts were performed in addition to epididymal sperm counts. Contrary to what was predicted, there was no significant difference in the number of sonication resistant sperm heads in the testis. This procedure is an assessment of the elongated spermatid population that is often lost in the purification procedure performed for flow cytometric evaluation. Experimentation was initially conducted on 12 old week animals, and according to the data that was collected using flow cytometry, there was no significant difference in the spermatid population at this age (figure 9). These findings were validated with the sonication resistant sperm head counts, and in reflection we should have initially selected either 8 or 16 weeks as the starting point in order to validate a significant decrease in elongated and round spermatids, which would tie together the two sets of experiments. However, subsequent elevation of daily sperm production at 16 weeks of age did not demonstrate a significant decrease in YHR\(^+\) compared to WT mice. Interestingly though, there were significantly
fewer sperm heads calculated in the YHR$^+$ animal for the pooled caput/corpus epididymis, but this was not corroborated at 16 weeks (figure 13). It is currently unknown if this is an artifact of the inherent variability with counting using a hemocytometer or if this is valid assessment. Another possible explanation for the lack of consistency between the flow cytometric data and sperm head counts could be due to too small of a sample size. Increasing the number of samples could potentially allow us to increase the stringency of the statistical tests for significance.

Hormonal support by both FSH and androgen are believed to play a critical role in both initiation of meiosis at puberty and in the maintenance of spermatogenesis throughout the adult life. Meiosis and differentiation of male germ cells is mediated through the Sertoli cell by virtue of the presence of FSH and androgen receptors. The absolute requirement of Sertoli cells for the progression of spermatogenesis is further evidenced by the lack of FSHR and androgen receptor in the germ cells. It is still unknown how the Sertoli cell conveys the necessary information to the germ cell in order to influence cell cycle progression. It was previously mentioned that FSH plays an important role in the development of immature testis by controlling the size of the Sertoli cell population, which is important in determining sperm output in adulthood. Beyond this, FSH plays an important role in promoting spermatogonial development, maintenance of key Sertoli cell functions, and the viability of meiotic and postmeiotic germ cells.

Based on the evidence that was generated, there was no significant difference in germ cell proliferation. Although it was hypothesized that there would be a decrease in germ cell proliferation due to a low concentration of FSH, the significant decrease in total...
germ cells and germ cell sub-populations that was calculated using flow cytometry can be 
explained another way. Because FSH has a diverse function in the Sertoli cell, it acts at 
multiple sites in the spermatogenic process and is required for quantitatively normal 
spermatogenesis (53). Further support for this exists from studies that have disrupted the 
FSHR or FSHβ subunit genes. In these studies FSH has been shown to be required for 
maintaining normal testicular size, seminiferous tubule diameter, sperm number and 
quality (53). The FSHβ subunit transgenic males were reportedly fully fertile despite 
having almost a 60% decrease in the number of mature spermatids and altered motility 
(64). These data indicate that although the YHR+ males have the same Sertoli cell 
numbers, and thus, the same infrastructure to support equal numbers of germ cells, a low 
concentration of FSH during adulthood is impacting the ability of the Sertoli cell to 
support a full complement of germ cells.

Contradictory to literature reports in animal studies that have manipulated either 
the gonadotropins or their receptors, the YHR+ animals show less apoptosis than the WT 
animals although no significance was recorded. It should logically follow that since 
YHR+ males have decreased concentrations of FSH that they should have an increase in 
apoptosis. More evidence is mounting that demonstrates that the withdrawal of trophic 
support causes an acceleration of germ cell apoptosis at specific stages of 
spermatogenesis. Spontaneous germ cell apoptosis naturally occurs in all three classes of 
germ cells (spermatogonia, spermatocytes, spermatids), although in mice it is most 
common in spermatocytes, and less common in spermatogonia and seldom seen in 
spermatids (65). The factors that trigger spontaneous germ cell apoptosis during normal 
spermatogenesis are not known at this time. It has also been proposed by Sinha Hikim, et.
al. (65), that apoptosis of germ cells can be grossly underestimated due to the rapid and efficient clearance of apoptotic cells. In order to fully hash out the apoptosis findings it will be necessary to increase the sample size in an effort to correct for variability that occurs with spontaneous apoptosis.

Because it was previously shown that YHR\textsuperscript{+} males are sub-fertile, one of the initial goals was to identify any changes in protein composition of epididymal sperm using two-dimensional electrophoresis. Although there were statistically significant changes in protein expression, reproducibility was poor, and therefore it was concluded that further investigation was not warranted. Challenges in identifying more differentially expressed protein spots may arise from the difficulty in solubilizing membrane proteins, inconsistencies in detecting low expression proteins, or minimizing the concentration of charged particles that are used in the lysis buffer. There is still the potential to extract information of changes that are occurring in spermatozoa as they undergo epididymal maturation. Narrowing the focus of our epididymal studies (purifying for specific proteins) or approaching it with a different technique (DIGE), might greatly aid in finding specific proteins that are changing and determining the significance of such alterations.

**Future Directions**

The results of this thesis provide evidence that premature activation of LHR alters qualitative and quantitative spermatogenesis. Based on the findings provided, there are a number of directions that this project could go in the next phase. First, the consequence of persistently low concentrations of FSH could be investigated by supplementing YHR\textsuperscript{+} animals with recombinant human FSH, such that these mice will have a level similar to
that seen in the WT animals. If this is able to restore qualitative and quantitative spermatogenesis then the focus can be narrowed to Sertoli cell signaling and/or possibly Sertoli cell-germ cell interaction and communication.

Second, in order to more precisely quantify the total Sertoli cell population, one would need to use an optical disector. Performing Sertoli cell counts as was done here in is good for providing relative numbers per tubule, but it does not accurately quantify the total population. In order to either support or dispute the proposal that the qualitative and quantitative decline in YHR⁺ germ cells is a result of the Sertoli cells not being able to support a full cohort of germ cells, we must able to accurately quantify the total germ cell population so the germ cell to Sertoli cell ratio can be determined. In addition to this, Sertoli cell function studies would need to be conducted in order to isolate any differences that may exist in the YHR⁺ animals.

Lastly, previously existing TUNEL stained sections were used in quantifying germ cell apoptosis for this study. Due to the difficulties in accurately staining and quantifying apoptotic cells, such as rapid and effective clearance of apoptotic cells by phagocytes (Sertoli cells), germ cell apoptosis can be underestimated (65). Based on this, the sample sizes of this study need to be increased and/or re-performed in order to increase their validity or provide significance to subtle changes.
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