SPERMATOGENESIS REQUIRES NUCLEAR ANDROGEN RECEPTORS IN SOMATIC CELLS AND TESTOSTERONE BINDING TO MEMBRANE RECEPTORS ON GERM CELLS IN RAT TESTIS

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BY

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DEDICATION

I am dedicating my dissertation to my parents, Mr. Amaresh Chandra Talapatra, and Mrs. Malaya Talapatra, my husband Mr. Samrat Moitra, and my sister Ms. Sangita Talapatra. Thank you for all your never-ending unconditional love, patience, and support.

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ABSTRACT

ARPITA TALAPATRA

SPERMATOGENESIS REQUIRES NUCLEAR ANDROGEN RECEPTORS IN SOMATIC CELLS AND TESTOSTERONE BINDING TO MEMBRANE RECEPTORS ON GERM CELLS IN RAT TESTIS
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Leydig cell ablation using ethylene dimethane sulfonate (EDS) triggers germ cell apoptosis by depleting testosterone (T) and subsequent loss of spermatogenesis. Male Sprague Dawley rats were injected with EDS at 75 mg/kg body weight to selectively eliminate mature Leydig cells and thus loss of testosterone. Two specific Leydig cell transcriptional markers (*3βHSD2* and *Insl3*), were quantified using qrtPCR and were depleted by >98% with Leydig cell loss that also resulted in complete testosterone loss in serum and testicular tissue and significantly increased germ cell apoptosis and significantly reduced testicular weight. Gene expression levels of pro-apoptotic genes and anti-apoptotic genes all in the Bcl2 protein family in testes were significantly increased 10 days post-EDS as measured by qrtPCR. Immunostaining for the nuclear androgen receptors (nARs) were found in Sertoli cell nuclei, myoid cell nuclei, and the stripped cytoplasm of spermatids in seminiferous tubules.

Using flow cytometry data from isolated germ cells tagged with Vybrant Dye Cycle (Green DNA dye) confirmed that haploid germ cells were mainly affected by loss of testosterone and this loss was prevented with testosterone replacement. Testosterone

act via both a canonical and a non-canonical pathway, primarily inducing nuclear androgen receptors (nARs) in somatic cells. However, maintenance of spermatogenesis requires testosterone activation of a non-canonical membrane receptor.

We investigated for existence of a functional non-canonical pathway that would be novel for testosterone action in testicular germ cells since testosterone and progesterone have similar affinity for receptor binding with membrane progesterone receptors. Transcription of five mPRs (α, β, γ, δ and ε) were found in male rat testes among which mPRs α and β had a more gene expression level and detection by immunostaining of protein expression. We further investigated whether testosterone binds mPRs in presence of low-level progesterone, mimicking normal testicular environment. We identified testosterone binding on isolated germ cells via IHC. Competitive binding assays were successful in binding testosterone with mPRs even in the presence of low-level progesterone. Thus testosterone may use an alternate pathway for receptor interaction on germ cells that may prevent apoptosis of differentiating germ cells of spermatogenesis.

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INTRODUCTION

Reproduction is the basic need for life. Sexual reproduction serves as the major foundation for the evolution of most species. Sexual reproduction requires involvement of two haploid gametes (germ cell) — sperm (male) and the egg (female). Normal healthy germ cells are required to produce a normal genetically distinct individual. Unlike the somatic cells, life cycle of the germ cells is different in many respects.

Germ cell fate specification during development has been a subject of concern. It is important to study how the identity of the cell influences its shape and morphology, and the process by which the biology of the germ cell regulates cytoplasmic determinants and asymmetric division. Germ cells were produced from cells possessing a localized cytoplasmic determinant, the germplasm. One of the most important aspects of germ cell specification was to repress somatic cell genes and reorder the cell towards germ cell program (Donoughe et al. 2014). Another common feature of germ cell is to migrate from their site of origin towards the site of maturation (Tarbashevich et al. 2010). Germ cells differentiation involves sex-specificity. Spermatogenic germ cells are genetically different from oocyte germ cells. Sex-determination in germ cells depends on the interaction of germ cells with their neighboring somatic cells (Barr, M. L et al. 1962). Germline stem cells can produce many gametes throughout a large portion of their life.

Throughout their life, germline stem cells interact with their niches to help maintain regulation of germ cell development (Fuchs et al. 2004).

Chromatin regulation is necessary within the nucleus and throughout the cell cycle for proper germ cell differentiation (Cunha et al. 2004). Germ cell gene expression is controlled by regulated epigenetics (Meng, X et al. 2000). Meiotic division of germ cells producing haploid set of chromosome shows unique chromatin characteristics regulating cell morphology during maturation (Yanowitz, J. et al. 2010). Scientists have also speculated that these chromatins, which are regulating germ cell differentiation, are protected by the piRNA pathway against transposable element mobilization (Bortvin 2013). Germ cell development is also associated with its death throughout the span in response to external or internal stimuli (Nantel et al. 1996).

Though germ cells are terminally differentiated cell types, fertilization of sperm and ova gives rise to a totipotent zygote which possesses the potential to form all type of cells in the body. Having a perception of the structural development and differentiation mechanism of different cell populations found during spermatogenesis can help in solving some of the mysteries related to germline stem cells and their potential therapeutic aspects.

Declining birth rate in developing countries is a significant social complication (ESHRE Capri Workshop Group. 2010). Reproduction is a natural experience for most couples. For some unfortunate couples, it is difficult to conceive since infertility has become a universal problem. Recent data suggests that about 15—20% of U.S.

demographics constitute non-reproductive couples. Forty to 50% of infertility accounts for male infertility, affecting 7% of all males (Kumar et al. 2015). Inability of a male to cause pregnancy in a fertile female due to his deficiencies in the semen is considered to be the root cause of male infertility (Cooper et al. 2009). Men face fertility problems due to low sperm count caused by problems in either sperm production or sperm transport. About two-thirds of infertile males have problems of sperm production due to chromosomal or genetic causes, infections, torsions, medicines, and chemicals or radiation damage. One in five infertile men has sperm transport problems due to infections or prostate related problems (Miyamoto et al. 2015). Aberration in the process of spermatogenesis is the main cause of producing potential sperm damage (O'Brien et al. 2010).

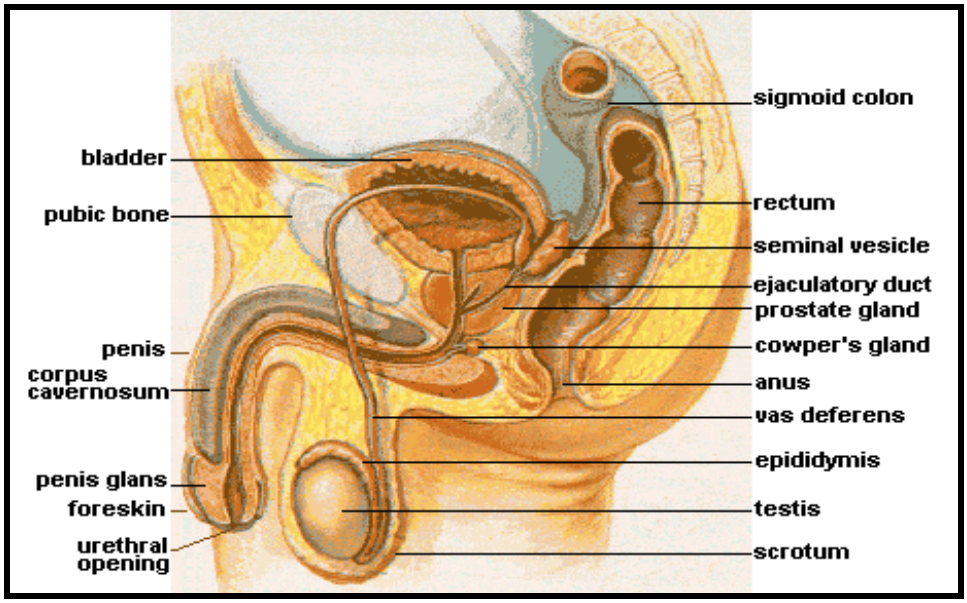
Due to lifestyle changes and advancement of technology, humans are often exposed to hazardous chemicals that are released into the environment at an alarming rate. A major turning point was the identification of these chemicals and naming them as endocrine disruptors (EDs; Diamanti-Kandarakis et al. 2009). Chemical - like polychlorinated biphenyles (PCBs; Pocar et al. 2011), polybrominated biphenyl ethers (PBDE; Abdelouahab et al. 2011), bisphenol A (BPA; Rubin et al. 2011), bisphenol S (BPS; Viñas et al. 2013), dichlor diphenyl trichloro ethane (DDT; Tiemann et al. 2008), and ethinyl estradiol (EE; Hogan et al. 2010) are some of the EDs that act as major regulators of male infertility. These EDs are known to act as imperfect ligands to nuclear and membrane bound receptors rendering interference of the hormone regulated cell

signaling pathways and gene expression (Marques-Pinto et al. 2013). Male infertility caused by EDs has resulted in reduced semen quality, urogenital tract abnormalities or testicular germ cell cancer which is termed as testicular dysgenesis syndrome (TDS) (Asklund et al. 2004). TDS often results from prenatal Leydig cell and Sertoli cell dysfunction due to low level of androgen and disabled germ cell development during spermatogenesis.

The male reproductive system consists of testis, its associated excurrent ducts (epididymis, ductus deferens and ejaculatory ducts) which help to conduct sperms, accessory glands (prostate gland, seminal vesicle, bulbourethral gland) and supporting structures like penis and scrotum. These reproductive organs are located outside the body, around the pelvis (see Figure 1).

Figure 1

The Human Male Reproductive Anatomy



The organs of the male reproductive system functions both as endocrine and exocrine system. It produces, maintains, and transports sperms as well as deliver them within the female reproductive tract. It also produces and secretes male sex hormones to regulate the functions of these organs (Tortora et al. 2006).

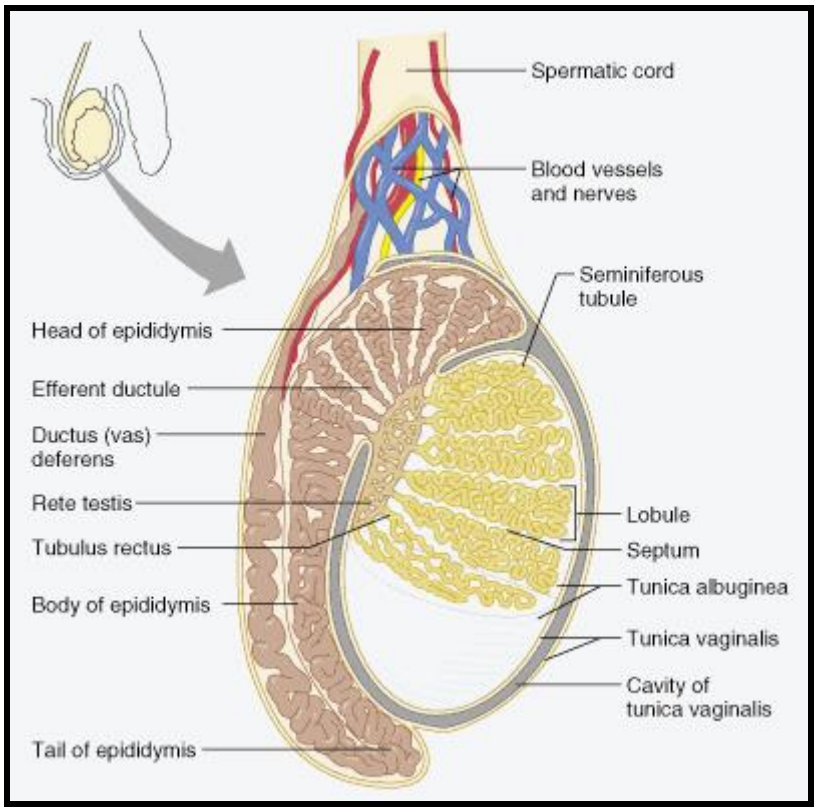
Male gonads, testes, are oval paired glands that lie in the scrotum secured by a structure called spermatic cord at either end. They produce sperms and hormones like testosterone.

Each testis is covered by three distinct layers of dense connective tissue capsule. The outermost layer, tunica vaginalis is a serous membrane covering the visceral portion of the scrotum (Leeson et al. 1962). The parietal layer of the tunica vaginalis lines the scrotum. The small amount of fluid present in between the parietal and the visceral layer acts as a lubricant between the scrotal wall and testis (Westen et al. 1992; see Figure 2). Tunica albuginea, a fibrous dense connective tissue structure, is composed of interlocking collagen fibers serve as the middle layer. The purpose of this smooth muscle layer is maintaining the capacity of the capsule for contracting on receiving pharmacological stimuli (Davis et al. 1970). The third and innermost layer consists of loose vascular connective tissues termed *tunica vasculosa*. It is rich in fine blood vessels and provides nutrition to the developing testis. The internal surface of tunica albuginea forms septa carrying blood vessels from underlying tunica vasculosa to enter towards a region called mediastinum within the testis. The anastomotic network of ducts is given a specialized name, *rete testis*. The connective tissue septae formed by the internal layer of tunica

albuginea divide the testis into series of internal compartments or lobules (Steinberger et al. 1975; see Figure 2).

Figure 2

Sagittal Section of Testis



Note. 2001 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc.

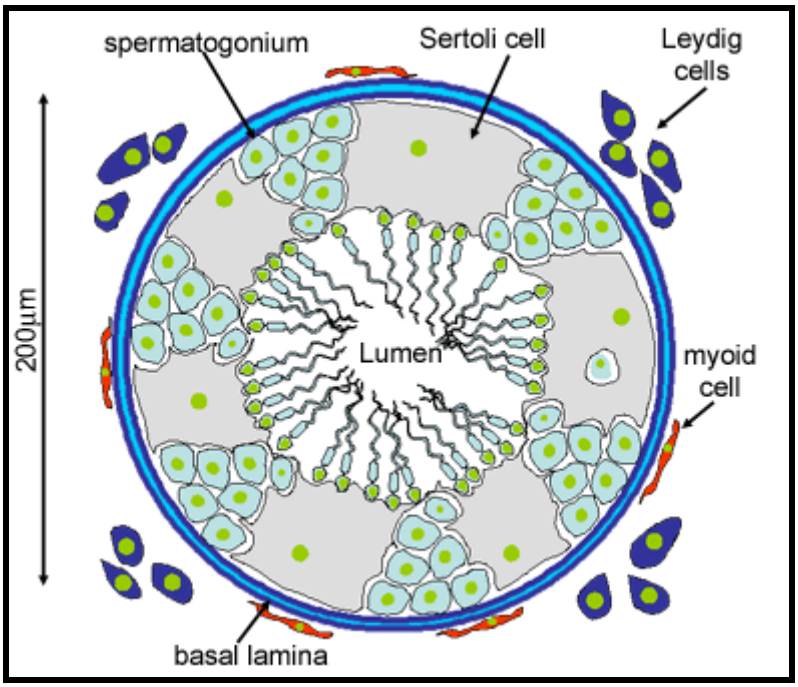
Testis can be divided into two functional units – seminiferous tubule and the interstitium. The interstitium serves as primary area for blood and lymph circulation. It is lined by perivascularly located interstitial cells (Leydig cells) along with occasional macrophages. These cells possess binding sites for luteinizing hormone releasing

hormone (LHRH) and luteinizing hormone (LH), which directly influences the functioning of these cells (Hsueh et al. 1979; Clayton et al. 1980). Abundance in smooth endoplasmic reticulum and mitochondria with tubular cristae in these cells participate in steroidogenesis, production of testosterone. Destruction of Leydig cells leads to reduced testosterone synthesis, which successively has a negative effect on testicular size and overall spermatogenesis process. It also retards the normal development of masculine characters (Griffin et al. 2004).

Seminiferous tubule, a highly coiled tubule, shows complex convoluted patterns. Both ends of the seminiferous tubule are attached to rete testis. Seminiferous tubule has many tight bends along with long straight sections. The portion which connects seminiferous tubule with intratesticular rete testis is known as tubulus rectus. Rat seminiferous tubule is composed of non-uniting complex convolutions but other species like human have anatomized tubules forming blind pouches (Steinberger et al. 1975). The wall of the seminiferous tubule from outside to inside is lined by endothelial cell layer, a layer of collagen, basement membrane, myoid cell layer, basement membrane, collagen layer, basement membrane and the cells of germinal epithelium. Sperm are produced inside this seminiferous tubule by the process of spermatogenesis (see Figure 3).

Figure 3

A single seminiferous tubule and cells in the interstitial tissue outside the tubule

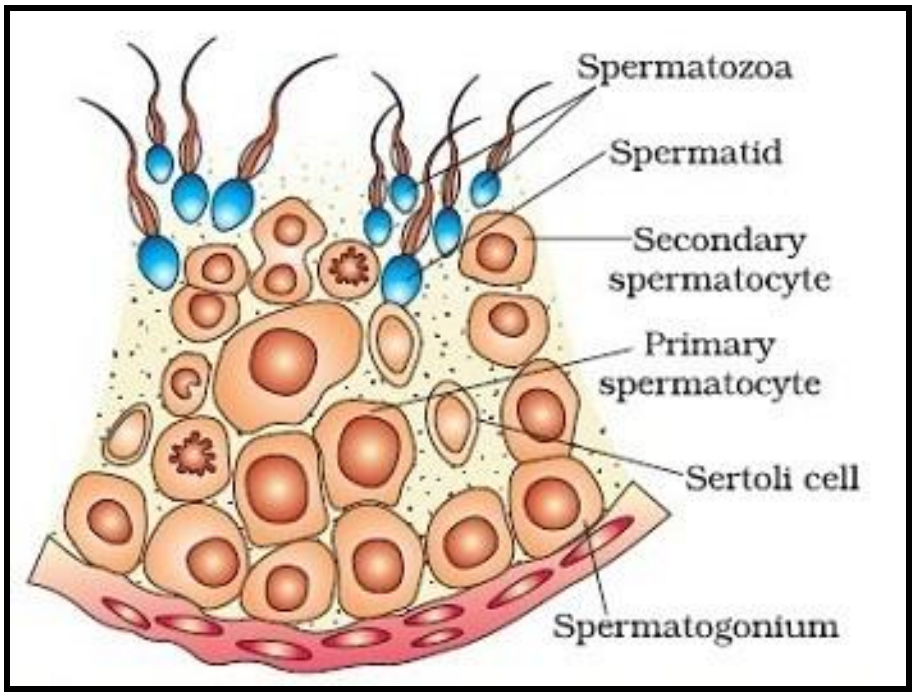


Note. The Histology Guide, Male reproductive system.

Cross section of seminiferous epithelium demonstrates developmental progression of germ cells from less mature cells at the periphery to more mature cells towards the lumen. At the base of the epithelium, spermatogonia are located followed by spermatocytes in the middle and spermatids near the lumen. Spermatids are spermiated here to form mature sperms that are released into the lumen (see Figure 4).

Figure 4

Transverse section of a portion of seminiferous tubule showing developmental progression of germ cells

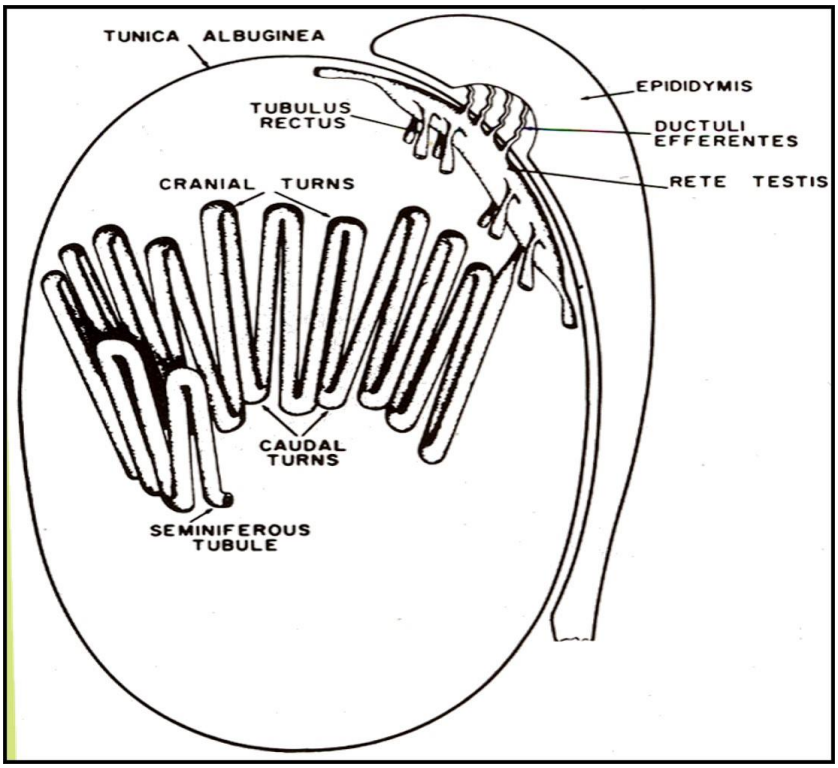


Note. Toppr answer Inc.

The intratesticular rete testis, lined by flat cuboidal epithelium, forms a narrow channel under tunica albuginea where it continues to form the extra testicular rete testis. This portion is closely coupled with the head of the epididymis through ductuli efferentes, series of channels emptying into the duct of epididymis. The extra testicular rete testis is rooted into the adipose tissues on the head of the epididymis (Steinberger 1975; Westen et al. 1992; see Figure 5).

Figure 5

View of seminiferous tubule and its connection to the rete testis.

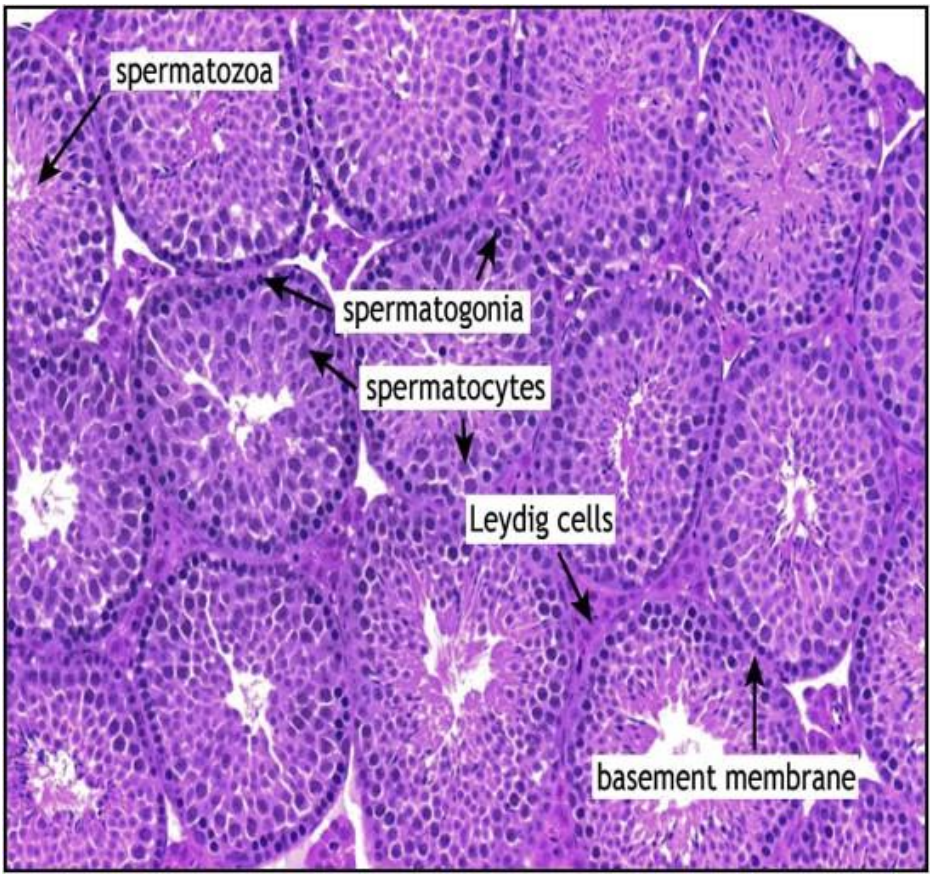


Seminiferous tubules comprise primarily of two types of cells — the primordial germ cells (PGCs) giving rise to gonocytes and Sertoli (sustentacular) cells — which supports and nourishes the growing spermatogenic cells.

Long lived germ cells responsible for spermatogenesis are gonocytes. They give rise to spermatogonia, which sequentially proliferates into primary and secondary spermatocytes, spermatids and spermatozoa. Spermatozoa then differentiate into mature sperms which are then released into the lumen of seminiferous tubule (see Figure 6).

Figure 6

Section of seminiferous tubule showing types of germ cells



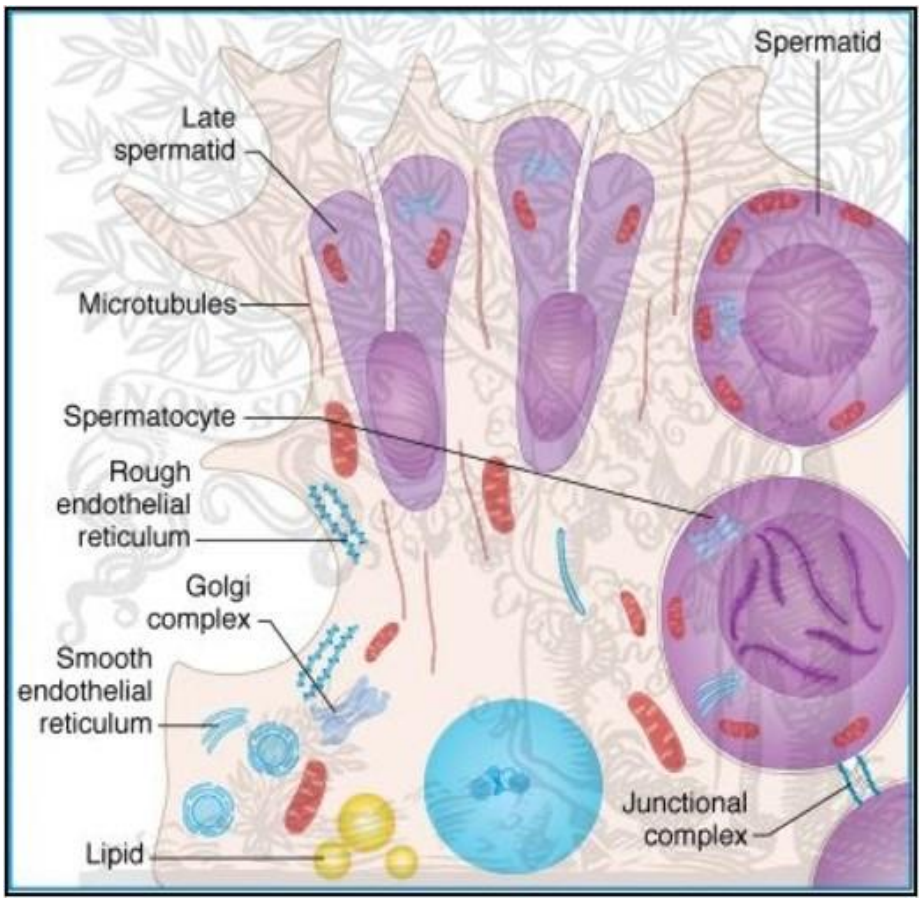
Note. Histology atlas. Male testis.

Successful spermatogenesis depends on the coordination of different cell types - Sertoli cells, Leydig cells, myoid cells. Sertoli cells determine expression of unique conserved genes that are responsible to carry out unique steps in spermatogenesis. They also directly and indirectly interact with the germ cells (Johnson et al. 2008; Russell et al. 1993).

Sertoli cells are the somatic components of the seminiferous tubule. It directly interacts with the developing germ cells. The 3D structure of Sertoli cells constantly change due to their proximity and interaction with the growing germ cells throughout the stages of development. They are columnar shaped cells located in basal to the adluminal compartment. Sertoli cells occupy 17—19% volume of the seminiferous tubule of an adult testis. Ultrastructure of Sertoli cells defines presence of long and thin mitochondria and lipid droplets at the base of the cytoplasm (Johnson et al. 2008; Russell et al. 1993). Nuclei of the Sertoli cells display variation in shapes; oval or pear-shaped nuclei show significant nuclear membrane depression. Sertoli cells have high metabolic rates that are evident by the presence of indented nuclear membrane, large distinct nucleolus, and euchromatin nucleoplasm. Large surface area of the Sertoli cells helps them to sustain greater number of developing germ cells. In adult rat testis, the Sertoli—germ cell ratio is about 1:50 (Wong et al. 1983). Maintaining this ratio is very important for its role in spermatogenesis (see Figure 7).

Figure 7

A single Sertoli cell with associated germ cells.

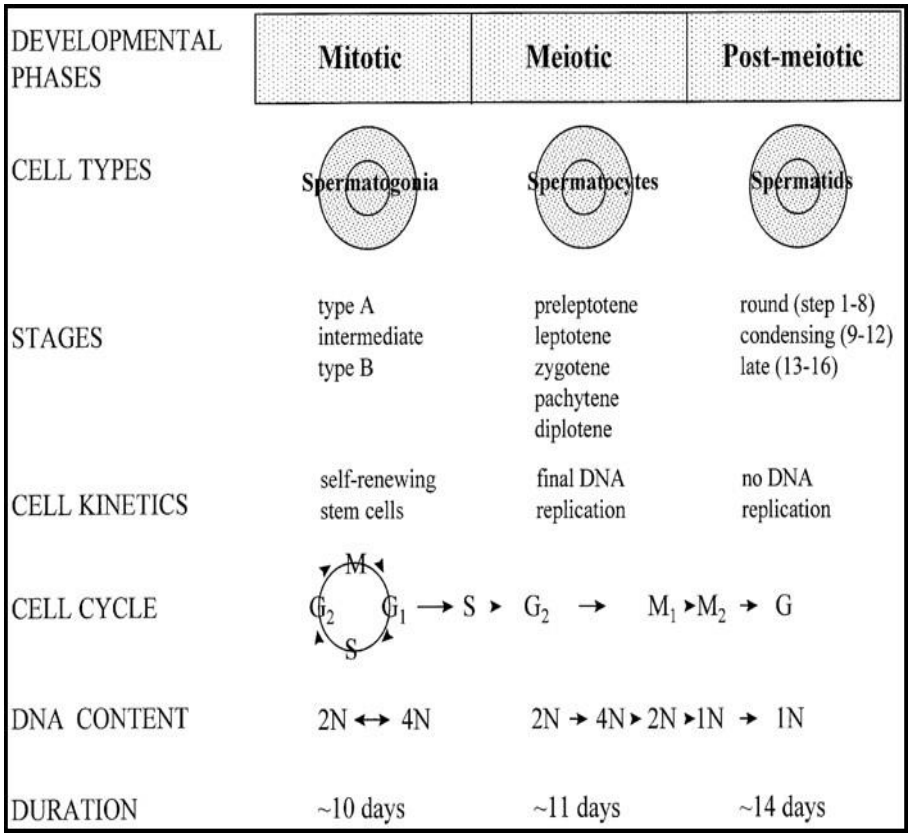


Morphologically, Sertoli cells are classified into two categories: Type A and Type B. Germ cells in certain stages of development (stage V in rats), remains deeply embedded into the cytoplasmic crypts of Type A Sertoli cells (Russell 1993). Type B Sertoli cells are more responsible for movement of elongated spermatids towards the lumen. During the stages of spermatogenesis, Type A Sertoli cells must alter themselves to Type B Sertoli cells to adjust themselves to the developing germ cells (Morales et al. 1993).

Spermatogenesis is the process of sequential cytological events occurring in precursor germ cells resulting in the formation of mature spermatozoa (Kerr et al. 2006). This process takes place within the seminiferous tubule throughout the reproductive phase of the male. In mammals and other vertebrates, developmental pattern of the germ cells occurs in overlapping waves accompanied by mitotic, meiotic, and post-meiotic germ cells that were developing during the course (Eddy et al. 1998; Perey, B et al. 1961; see Figure 8).

Figure 8

Developmental process of spermatogenesis

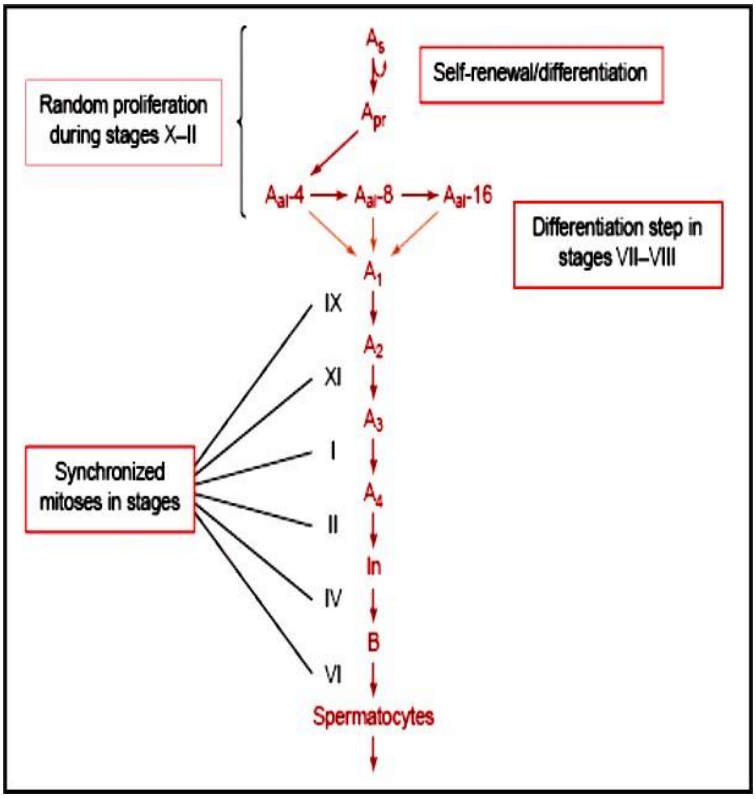


Processes unique to the germ cells (like meiosis, genetic recombination, haploid gene expression, formation of the acrosome and flagellum, remodeling and condensation of chromatin by protamines after replacing histones, and remodeling the nuclear lamins) must occur at the precise time to produce a viable gamete (Eddy et al. 2002). Within the seminiferous tubule, there is a series of successive cellular association appearing in any one area. Based on the intrinsic and extrinsic signaling within the germ cells and differential influence of Sertoli cells throughout distinct developmental stages, the process of spermatogenesis can be interrupted or subdivided into series of distinct phases. As the discharge of spermatozoa can be viewed as the secretory product of the process, the process involves continuous replication of stem cells to produce batches of cells that carry on the simultaneous changes (Lee et al. 2009).

Germ cell precursors (spermatogenic stem cells) are required for continuous supply of germinal epithelial cells that undergo proliferation to form mature germ cells. The epiblast cells give rise to the PGCs that in turn give rise to spermatogenic stem cells (De Rooij et al. 1998; see Figure 9).

Figure 9

Scheme of spermatogonial multiplication and stem cell renewal in mice, showing the subsequent spermatogonial cell types and some cell kinetic characteristics of these cells)



According to studies on mouse PGCs, Resnick et al. (1992) observed that under definite conditions, single celled PGCs can form clusters of cells resembling the undifferentiated embryonic stem cells. PGCs furthermore divide to form gonocytes, which proliferates and arrests itself at the G_0/G_1 phase of the cell cycle until birth (Resnick et al. 1992).

In rats, length and diameter of the seminiferous epithelium starts to increase shortly after birth. This process continues until it reaches its adult size along with spermatogenesis (Steinberger et al. 1975). Rapidly after birth, most of the gonocytes in the seminiferous epithelium degenerates and the remaining few starts dividing postnatally on the fourth or fifth day to form diploid germ cells. Diploid germ cells constitute the first phase of spermatogenesis. These diploid cells, known as spermatogonia, are the most immature cells positioned at the base of seminiferous tubule. They multiply mitotically to continually replenish the germinal epithelium. Having a capability of self-renewing, they produce basally — located stem cells and committed cells that are destined become spermatozoa. Stem cell renewal in testis has been the topic of controversy for decades. To account for explaining the mechanism of stem cell renewal, two theories stand out of the pool of different explanations. Clermont and Bustos-Obregon (1968) proposed the earliest model, the A_0 model. A_0 model divides testicular Type A spermatogonia into five discrete classes: A_0, A_1, A_2, A_3, and A_4 (Clermont et al. 1968). The actively proliferating stem cells (i.e. A_1-A_4 cells) are termed as renewing stem cells. Not only do they give rise to mature germ cells, but they also still retain their pluripotency which means the A_4 spermatogonia can differentiate to form A_1 and In (Intermediate) spermatogonia. A_0 spermatogonia are the reserve stem cells that remain dormant during normal condition but start differentiating and divide to form renewing stem cells during the time of threat for the germinal epithelium.

The second model, A_S model, proposed by Huckins and Oakberg in 1971 (Steinberger et al. 1975), was validated and accepted by other evidence (Dupressoir et al. 1996; Van der Meer et al. 1992; Van der Meer et al. 1992). This model proposes three distinct classes of Type A spermatogonia: A_{single} (A_s), A_{paired} (A_{pr}), $A_{aligned}$ (A_{al}). This classification is based on their arrangement on the basement membrane. The A_S stem cells undergo either symmetric (produces either A_S cells to preserve the stem cell count or A_{pr} cells, which are connected by intercellular bridges) or asymmetric division (one of the daughter cell produces A_S cells and the other one produces A_{pr} cells). The A_{pr} cells produced from the asymmetric division differentiate to form four A_{al} spermatogonia, which further divides to give rise to chains of eight or 16 or infrequently 32 A_{al} spermatogonia (De Rooij et al. 1998). Series of six divisions produces A_1, A_2, A_3, A_4, intermediate (In), and B spermatogonia resulting in primary spermatocytes (de Rooij et al. 2001).

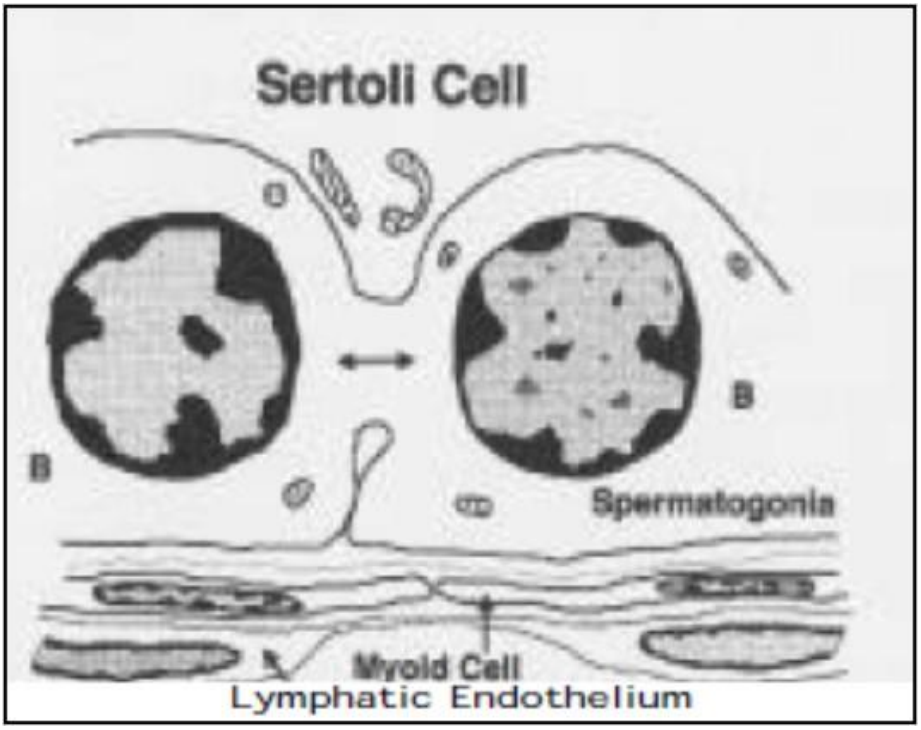
In rats and mice, there are four classes of spermatogonia are present: (a) Undifferentiated Type A spermatogonia {A_{single} (A_s), A paired (A_{pr}), and A aligned (A_{al})}; (b) Differentiated Type A spermatogonia {A_1, A_2, A_3, A_4}; (c) Intermediate spermatogonia (In); and (d) Type B spermatogonia (B; de Rooji et al. 2000; Russell et al. 1993). Different classes of spermatogonia are differentiated by the presence and distribution of heterochromatin (Chiarini & Russell. 2001). The undifferentiated spermatogonia including the stem cells (As) are regulated by Sertoli cells and occupy the niche of the seminiferous epithelium (Chen et al. 2005; Hess et al. 2006; Ogawa et al.

2005; Ryu et al. 2003; Ryu et al. 2006). Testosterone along with vitamin A modulate transcription of genes such as Glial cell line-derived neurotrophic factor (GDNF), TGF-β super-family proteins like GDNF family receptor α1 (GFR α1), promyelocytic leukemia zinc-finger (PLZF; Hess et al. 2006), c-*kit* receptor and its ligand stem cell factor (SCF; Mauduit et al. 1999; Schrans-Stassen et al. 1999) regulate the proliferation and differentiation of A_S spermatogonia. Guan et al. (2006) reported that stem cells extracted from mice when grown in vitro, it differentiated into different visceral organs specialized cells like that of the heart, brain, and skin. When these cells were transferred into early embryos, they helped in organ development (Guan et al. 2006).

Type B spermatogonia (Hess 1999; see Figure 10) divide mitotically at the end of the first phase to produce the first cells of the second phase, the preleptotene spermatocytes. These cells then migrate upwards (i.e., away from the base and cross the Sertoli-Sertoli junction).

Figure 10

Two type B-spermatogonia are connected through intercellular bridge



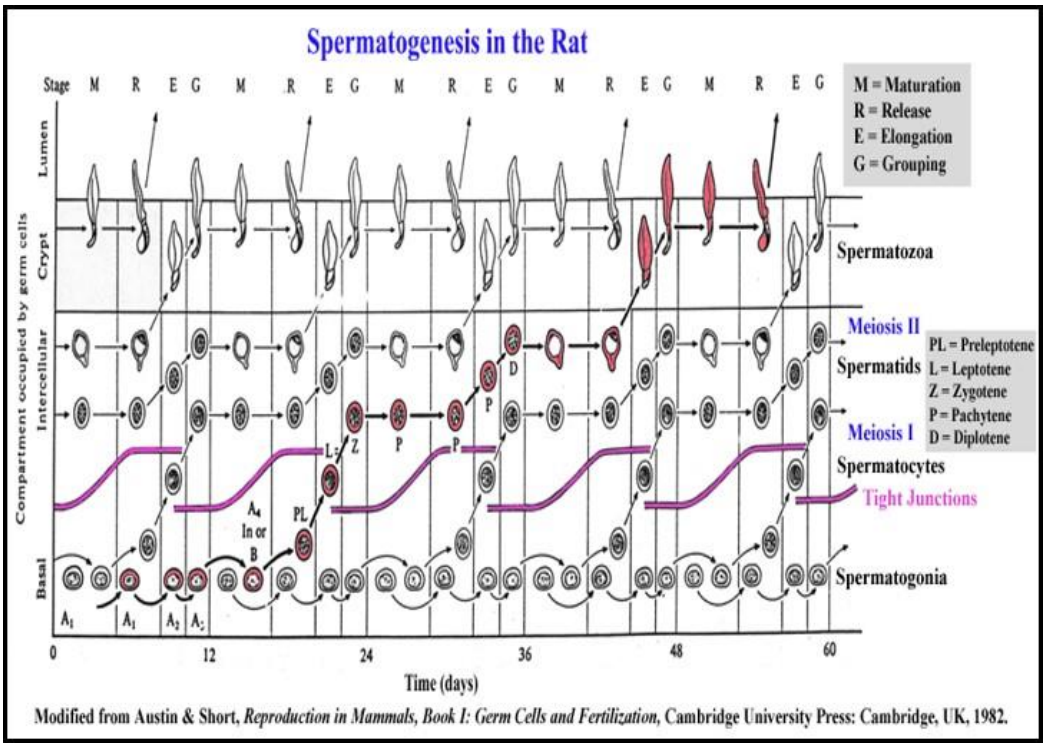
Spermatocytes ranging from RBC sized preleptotene to larger pachytenes constitute the second phase of spermatogenesis, reduction division by meiosis. Changes occurring in the dividing germ cells in this phase increase in DNA content and divide twice to produce haploid (half the number of chromosomes) cells.

Prophase of the first meiotic division lasts for about 3 weeks. Preleptotene spermatocytes start DNA synthesis. The chromosomes are untangled and appear as thin filaments in the leptotene stage. During zygotene, synaptonemal complex is formed by pairing of the homologous chromosomes. Massive changes appear during the pachytene stage. As the chromosomes shorten and thicken, nucleus enlarges enhancing the size of

the cell. Cross-over occurs between the paired chromosomes resulting in genetic recombination. RNA and protein synthesis start in this stage of division preparing the cell for reduction division. In the diplotene stage the synaptonemal complex is separated and the chromosomes are spread apart in the nucleus. Until condensation of the chromosomes take place in the diakinesis stage. At diakinesis, nuclear envelop also disappears. The two stages of meiotic division occur promptly resulting limiting the cells at one stage. After meiosis I, small diploid secondary spermatocytes are formed, which undergo division (meiosis II) to form very small haploid round spermatids. These haploid cells enter the third phase of differentiation (Hess et al. 2009; see Figure 11).

Figure 11

Cycle of spermatogenesis in rat



Note. Modified from Austin & Short, Reproduction in Mammals, Book I: Germ cells and fertilization, Cambridge university press: Cambridge, UK, 1982.

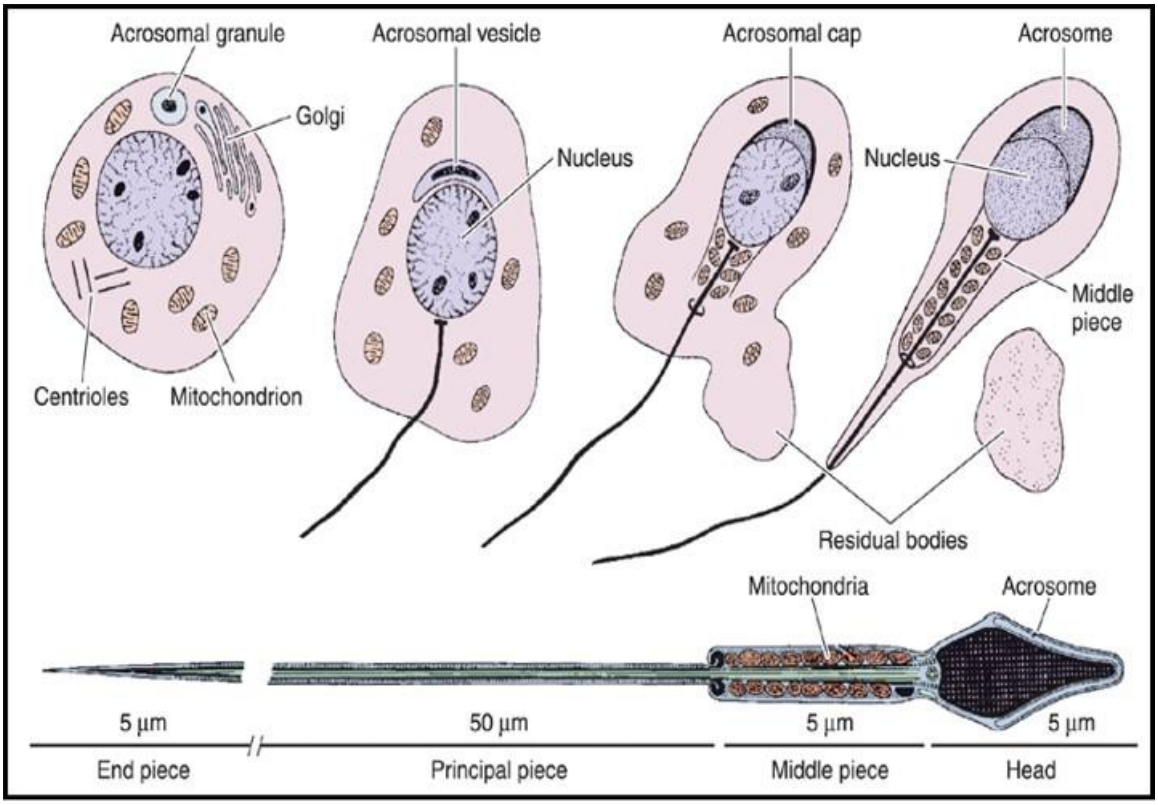
The cells in meiotic division occur in the single stage, XII. Studies in mice revealed that the duration of meiotic division is approximately 1 day. Cross-sectional studies detected stage XII covers only 10% of the seminiferous tubule (Hess et al. 1998). In species like rats, buffalos, rams, and pigs, the size of primary spermatocyte increases massively from preleptotene to diplotene (França et al. 1998; França et al. 2005). Post inflation, there is a dramatic decrease in cell size during spermiogenesis. This reduction

in size is due to nuclear and chromatin condensation. Nuclear size reduces about 50 times its original volume.

Haploid round spermatids experience an extended final phase of differentiation transforming them to highly condensed mature spermatozoa by the process known as Spermiogenesis. These haploid germ cells are subjected to substantial changes and modifications. Marked changes include (a) elongation of nucleus; (b) condensing of the chromatin into very dark staining structure; (c) production of lysosomal like granules by the Golgi apparatus that covers the nucleus to form the future acrosome, these granules are major sources of hydrolytic enzymes required for sperm-egg interaction and fertilization; and (d) formation of a tail like structure containing proximally located elongated mitochondria, it gets rid of any extra cytoplasm which is phagocytized by the Sertoli cells as residual bodies (Baker et al. 1976; see Figure 12). Spermatid differentiation can be divided into four extended stages or “steps.” These steps are necessary for identifying the changes that occur in the seminiferous epithelium.

Figure 12

The principle changes occurring in spermatid during spermiogenesis



At the preliminary steps of spermiogenesis Golgi apparatus plays a key role in the formation of acrosome. The acrosomal cap indicates the anterior or head of the future sperm. It is rich in glycoprotein and consists of enzymatic components secreted from the vesicles and granules of the Golgi. The first 3 steps of spermiogenesis are marked with

the presence of a prominent Golgi apparatus identified by PAS staining (stains the Golgi) and hematoxylin-eosin (HE) staining (stains nucleus).

Step I starts with division of secondary spermatocytes into spermatids. The Golgi zone or idiosome is lightly stained by PAS and is located close to the nucleus. HE stain shows condensed dark bluish black sickle shaped nucleus with indistinct tail filament. Angle between the caudal and apical extremes is 90˚ (see Figure 13 A).

Step II initiates with the formation of pro-acrosomic granules in the idiosome. Single small granule is usually centrally located. Occasionally two to four granules may be observed. Eosin deeply stains the middle piece making the tail clearly visible. Nucleus turns pale grayish. It also loses curvature. Angle between the caudal and apical extremes becomes greater than 90˚ (see Figure 13 B).

At Step III, fusion of the proacrosomic granule leads to the formation of a single large spherical acrosomic granule. The surface of the granule is flattened at the nuclear side. In spermatogonia are located at the basement membrane. Oval shaped nucleus of In-spermatogonia contains deeply stained chromatins along the nuclear membrane (Clermont et al. 1957; see Figure 13 C).

The Cap phase involves Steps IV-VII. At Step IV, due to flattening at the surface of the nuclear membrane, the acrosomic granule acquires a hemispherical shape. As the acrosomic granule spreads laterally over the nuclear surface, the head cap starts appearing. The total span of the acrosomic system (acrosomic granule along with head cap) should not exceed twice the size of the granule alone. Starting from this stage, the

idiosome remains associated with the acrosomic system until the end of the capping phase. In-spermatogonia divides to produce type B spermatogonia containing large clumps of chromatin in the inner surface of the spherical nucleus, which can be identified by dark HE staining (see Figure 13 D).

Step V marks the expansion of the acrosomic system greater than twice the size of the granule alone. As the acrosomic system covers a quarter of the nuclear circumference, this stage is terminated. Only heavily stained type B spermatogonia are present during this stage (see Figure 13 E).

During Step VI, the circumference of the nuclear outline is covered more than a quarter by the acrosomic system. Side view of the cell shows a distinctly grown head cap. Towards the end of the stage, the head cap covers one third of the nuclear circumference. Type B spermatogonia differentiate to form resting spermatocytes. As these spermatocytes move backwards towards the lumen, maturing spermatids are located towards the center of the tubule. Nuclei of the resting spermatocytes are identical with type B spermatogonia but are one-third of its size (see Figure 13 F).

The acrosomic system covers half the nuclear surface in Step VII. The polyhedral cytoplasm still contains a centrally located nucleus. The maturing spermatids are lined on the surface of seminiferous epithelium as they lack their bundle arrangement. Cytoplasm of these spermatids contains large basophilic bodies near the nucleus. During interphase, the newly formed spermatocytes are located along the basement membrane (Clermont et al. 1957; see Figure 13 G).

Acrosomal phase involves Steps VIII-XIV during which the acrosomal system moves over the ventral surface of the nucleus. The morphology of these steps is characterized by the side view of the nucleus.

During Step VIII, the haphazardly located spherical nucleus comes in proximity with the cytoplasmic membrane. The part of the nucleus covered by head cap becomes slightly depressed. The maturing spermatids along the surface of seminiferous epithelium release their excess cytoplasm as residual bodies (see Figure 13 H).

Step IX can be identified by an elongated and laterally flattened nucleus. The depressed part of the nucleus covered by the head cap is protruded on one side indicating the direction of elongation. The older generation of maturing spermatids transforms into spermatozoa and leaves the seminiferous tubule. The growing generation of spermatocytes enters the meiotic prophase and remains at leptotene stage (see Figure 13 I). With the emergence of Step IX, complex morphological changes occur in the spermatid nucleus. The sharp tip or apex is acquired along with a small concave surface or base (caudal extremity) at the opposite end where the tail gets attached. The concave side of the nucleus becomes the ventral surface whereas the convex side becomes the dorsal edge. Ventral angle is defined as the angle between the base and ventral surface while the angle between the base and dorsal edge, becomes the dorsal angle. The lateral surfaces are designated at the two flanks of the nucleus (Clermont & Perey 1957; see Figure 14).

The spermatids shift to its next step, Step X, with a clearly visible ventral angle. The cells become elongated with distinct lateral flattening. Towards the end of this stage, the dorsal angle becomes distinctly rounded (see Figure 13 J).

Sharp outlined ventral and dorsal angles emerge at the onset of Step XI. The apical part of the elongated nucleus is covered by the head cap and starts to take a tube-like structure. The acrosome appears as a crest at the apical end of the prominent dorsal edge (see Figure 13 K).

During Step XII, the acrosome reaches the apex of the nucleus taking a triangular structure. Having lost the dorsal edge curvature, nucleus bents itself like a hook. Condensed chromatins can be identified as a darkly stained structure (see Figure 13 L).

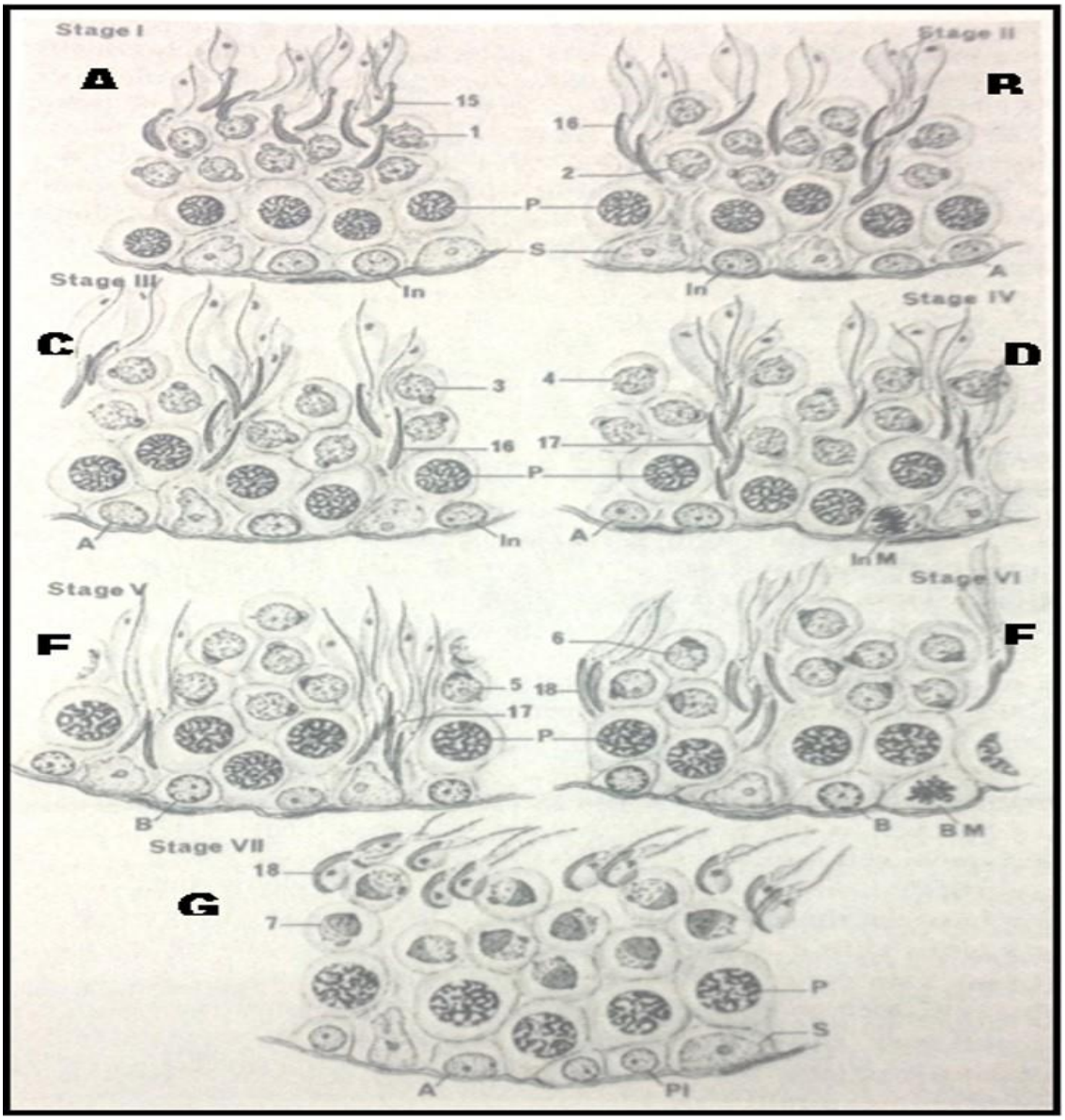
Step XIII begins when the tip of the spermatids becomes pointed. Elongated acrosome moves posterior along the dorsal edge. Chromatin condenses more and as a result the head cap releases its pressure on the nucleus and floats freely on the ventral side. The part of the nucleus covered by the head cap prominently bends ventrally (see Figure 13 M).

The final step of this phase, Step XIV, is identified with the existence of the maturation division of primary and secondary spermatocytes until the secondary spermatocytes are completely divided. The spermatids in this step show a marked decrease in nuclear length. The head cap is not supported by the nucleus. The acrosome forms a crest on the dorsal edge. The mitochondria concentrate around the flagellum.

Neck or the connecting piece is formed when the centrioles move back to the nucleus (Clermont 1972; Clermont et al. 1957; see Figure 13 N).

Figure 13 A-N

Illustrations of 14 stages of seminiferous epithelium cycle of rats as seen in PA Schiff-hematoxylin stained sections of testis



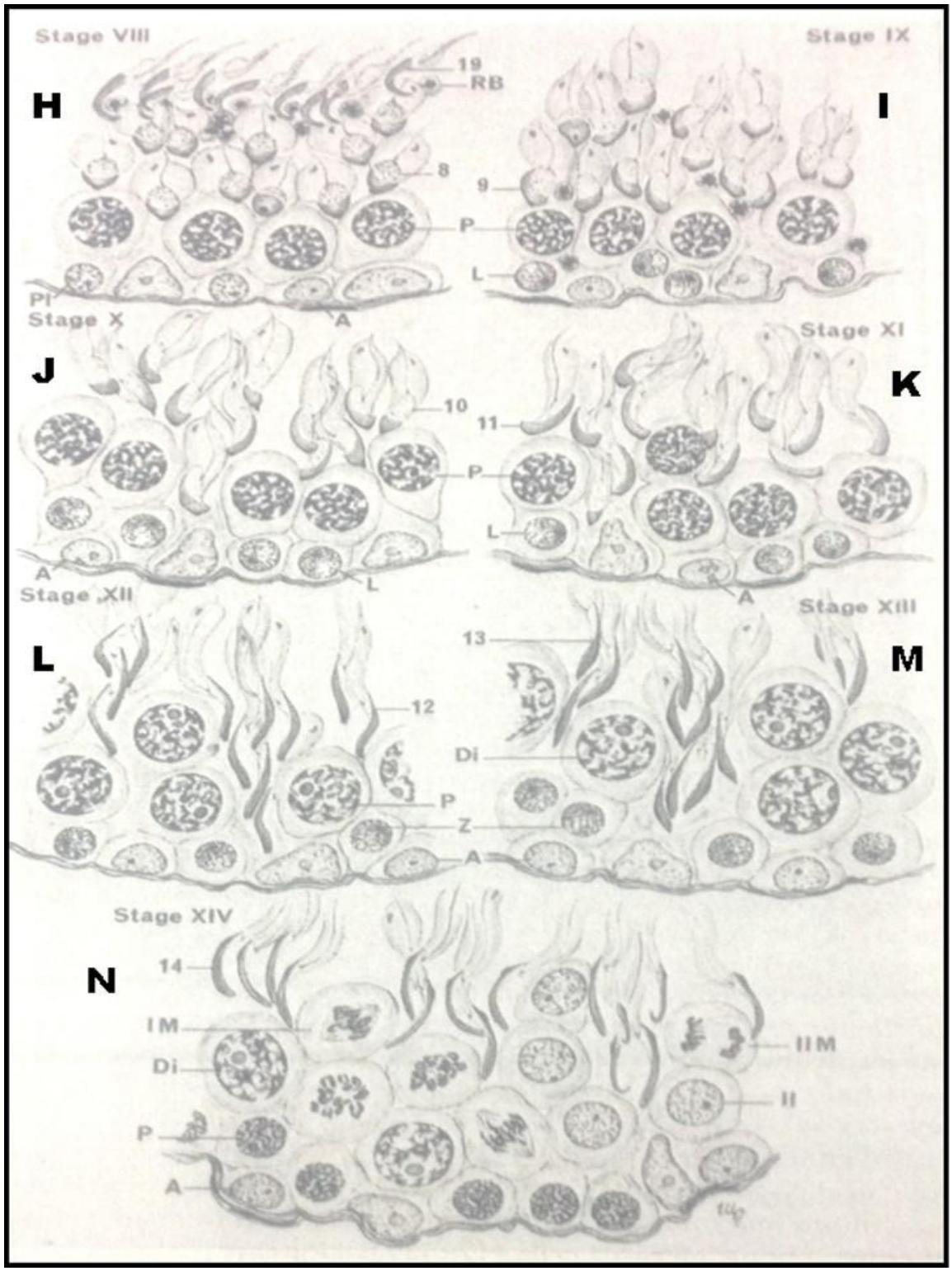
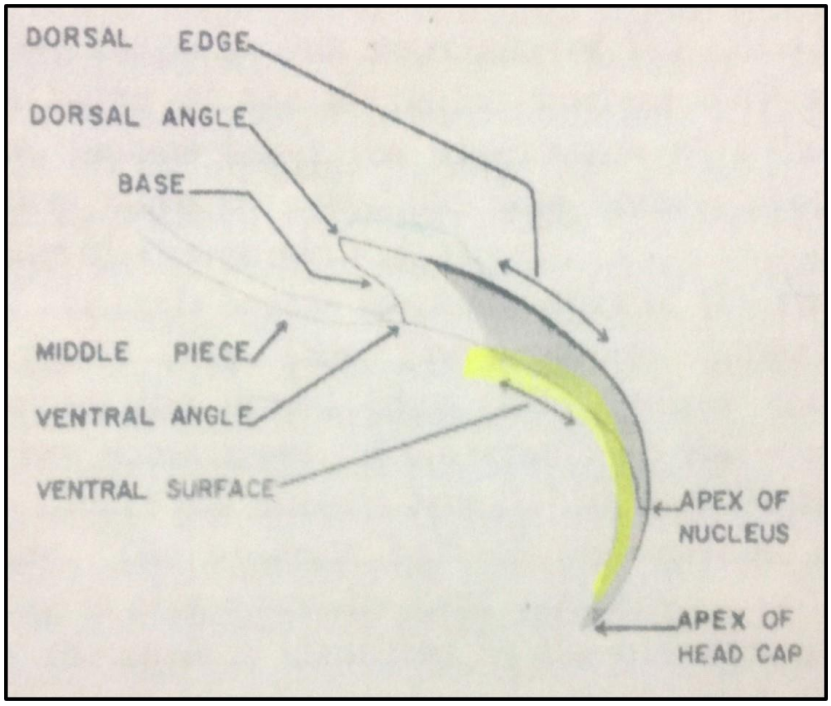

Stage VIII
H
19
RB
8
Pl
A
Stage IX
I
9
P
L
Stage X
J
10
P
A
L
Stage XI
K
11
P
L
A
Stage XII
L
12
P
Z
A
Stage XIII
M
13
Di
Stage XIV
N
14
IM
Di
P
A
IIM
II

Figure 14

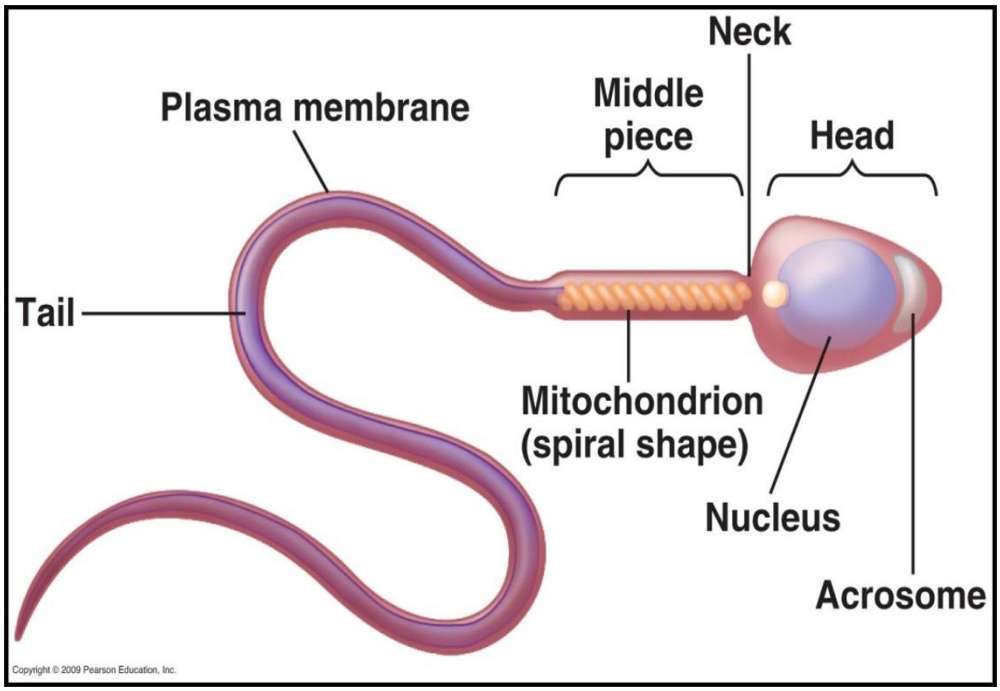
Diagrammatic view of the head of rat's spermatozoon seen from right lateral surface



The final phase of spermiogenesis involves changes resulting in maturation of the spermatozoa. The mature spermatids are released into the lumen as spermatozoa or mature sperms. The cytoplasmic residual bodies (containing unused mitochondria, ribosomes, lipids, vesicles, etc.) shed off by the developing spermatocytes are phagocytosed by the Sertoli cells. The mature spermatozoa are devoid of cytoplasmic ribosomes but contain only the mitochondrial ones. In spermatids, mitochondria are highly vacuolated, which changes to a crescent shape in spermatozoa (see Figure 15).

Figure 15

Morphology of a mature spermatozoon



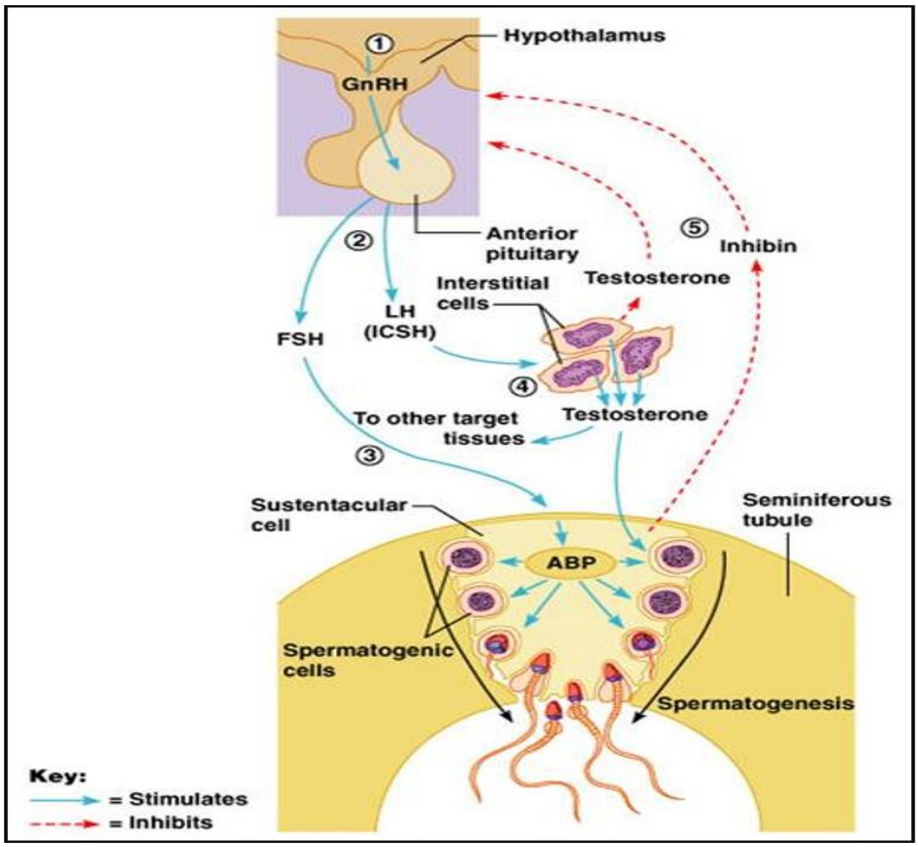
Note. 2009 Pearson Education, Inc.

Regulation of spermatogenesis occurs at three levels: interactive, intrinsic and endocrine. Intrinsic regulation maintains a conserved specific and unique gene expression for orchestrated progression of spermatogenesis. Interactive regulation is brought about by the Sertoli cells as it possesses androgen receptors and process the downstream signaling pathways to elicit the effect on the developing germ cells. Endocrine regulation is controlled by the endocrine signals mediated by hormones secreted by anterior pituitary (LH and FSH) and Leydig cells (testosterone), which in turn directly affect the Sertoli cells and indirectly the gene expression of germ cells.

Endocrine action regulating spermatogenesis depends on the collaborative effect of the action of pituitary gonadotrophins like LH, FSH, and steroid hormone like testosterone, androstenedione, and dehydroepiandrosterone (see Figure 16).

Figure 16

Endocrine regulation of spermatogenesis



Note. Austin community college, Reproductive system.

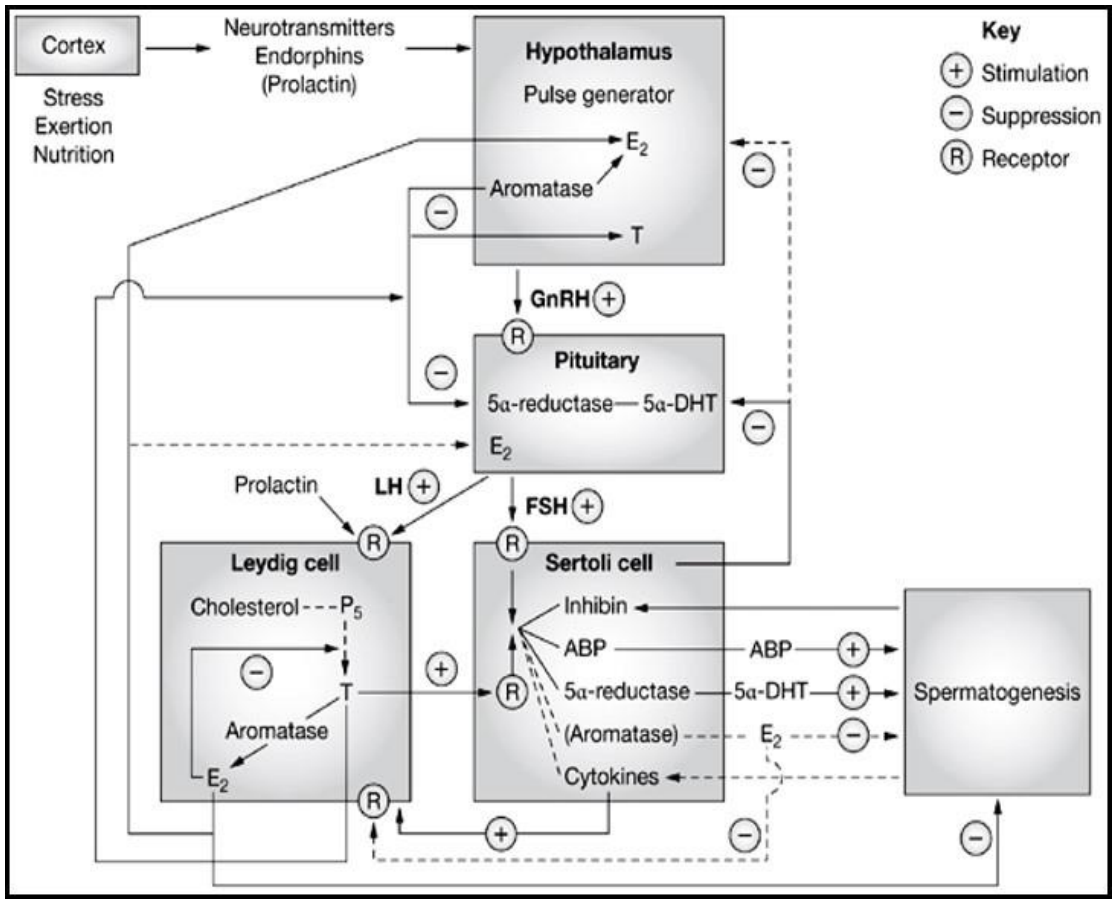
FSH produced by anterior pituitary increases LH receptors on Leydig cells (Sluka et al. 2006). LH from the anterior pituitary increases cholesterol desmolase activity in Leydig cells resulting in testosterone (T) production (Stocco et al. 2006). Testosterone

binds to the androgen receptors (AR) on Sertoli cells to modulate necessary signals for spermatogenesis.

T, a lipophoilic steroid hormone, acts as the primary steroid hormone in males. It is the major androgen that regulates spermatogenesis (Sharpe et al. 1994). Many genes regulated by testosterone are identified in testis within which Sertoli cell specific genes share a small number. Microarray studies revealed that 65% of the AR-regulated genes in testis are linked with conserved androgen response element (ARE) promoter (McLachlan et al. 2002). Withdrawal of T or knockdown of ARs resulted in disruption of the process of spermatogenesis by disintegrating blood testis barrier (BTB), production of low-quality sperms, etc., due to high amount of germ cell death of germ cell apoptosis (see Figure 17).

Figure 17

Regulation of spermatogenesis

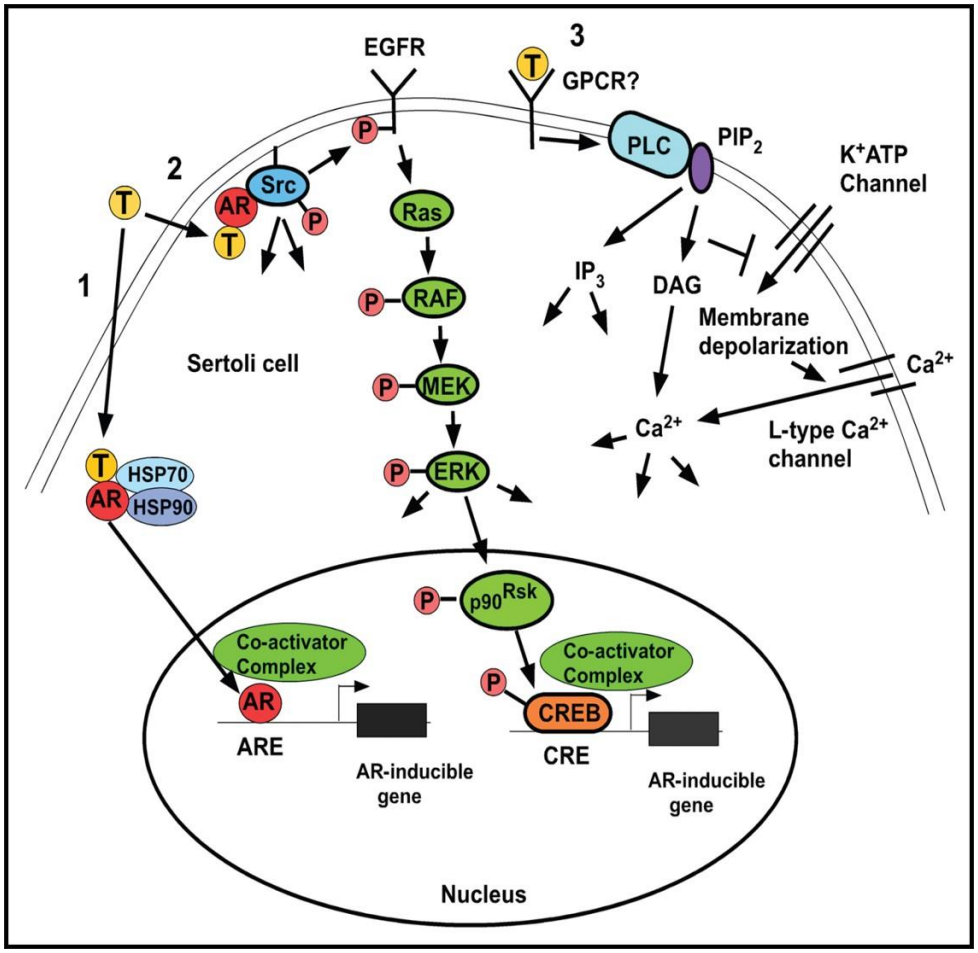


Action of testosterone takes place via two pathways: the classical pathway and the non-classical pathway (Walker et al. 2009; Walker et al. 2010). In the classical pathway, steroid hormone testosterone diffuses through the plasma membrane and binds with the AR sequestrated by the HSPs present in the cytoplasm. Ligand binding to the AR changes its conformation and it is released from its complex with HSPs. The AR-T complex is translocated in the nucleus where this complex acts as a transcriptional

regulator by binding with the ARE located upstream of the target gene promoter. Thus, it can regulate recruitment of co-regulator proteins and target gene transcription. Gene products of these genes can function in different stages of spermatogenesis. Changes in gene expression can be activated at a time interval of 30-45 mins after this pathway is activated (Shang et al. 2002; Walker 2011; see Figure 18 - 1).

Figure 18

Testosterone Signaling Pathways (1) The Classical Testosterone Signaling Pathway (2) The Non-Classical Kinase Activation Pathway



A large number of genes regulated by testosterone are identified in testis within which Sertoli cell specific genes share a small number. Microarray studies revealed that 65% of the AR-regulated genes in testis are linked with conserved ARE promoter. Rhox5 (Pem) homeobox Transcription Factor encoding gene was found to be induced in Sertoli Cells through ARE promoters (Lindsey et al. 1996). Completion of spermatogenesis thus may not be dependent on single a single AR-regulated gene but on the collaborated effect of the mutations in multiple AR-regulated genes (Zhou et al. 2010).

Non-classical mechanism of testosterone action occurs in two different phenomena. In the Calcium Influx Pathway, T activates L-Type Ca^{2+} channels present on the Sertoli cells and readily starts influx of Ca^{2+} ions into the cells. The former also activates G_q type G protein coupled receptor which stimulate Phospholipase C (PLC) to hydrolyze membrane bound PIP_2 into IP_3 and DAG. As PIP_2 antagonistically regulates K_+ ATP channels. When PIP_2 level is decreased, K_+ ATP channels are closed to increase membrane resistance and cell depolarization (Von Ledebur EI et al. 2002; see Figure 18-3).

In the Src Tyrosine Kinase Pathway, triggering of Sertoli cells by T localizes ARs in the plasma membrane. Proline rich motifs in ARs then interact with the SH3 domain of Src tyrosine kinases thereby activating it (see Figure 18-2). Activated Src mediates intracellular signaling pathway to phosphorylate EGF receptor (EGFR) present on Sertoli, Leydig and peritubular cells resulting in activation of MAP kinase signaling pathway involving Raf, MEK, and ERK. This cascade of reaction causes phosphorylation

of CREB transcription factor by $p90^{Rsk}$. CREB-TF translocate in the nucleus to induce CREB mediated gene expression. EGF precursors are present in pachytene spermatocytes and round spermatids rendering their function in cell survival (Fix et al. 2004). Src also phosphorylates components of adhesion complex (Focal Adhesion Kinase (FAK), β-Catenin, and N-Cadhenin) and contribute towards cell adhesion between Sertoli cells and mature elongated spermatids (Kinch et al. 1995; Roura et al. 1999; Xia et al. 2005).

Retinoid receptors RARα and RXRβ are nuclear receptors critical for spermatogenesis majorly for type A spermatogonia (Kim et al. 1996). RARα occur in spermatogenic cells (Wang et al. 1993) whereas RXRβ are present in Sertoli cells (Kastner et al. 1996). RTR or GCNF receptors are also present in round spermatids but there is no known Ligand or function for these receptors (Chen et al. 1994).

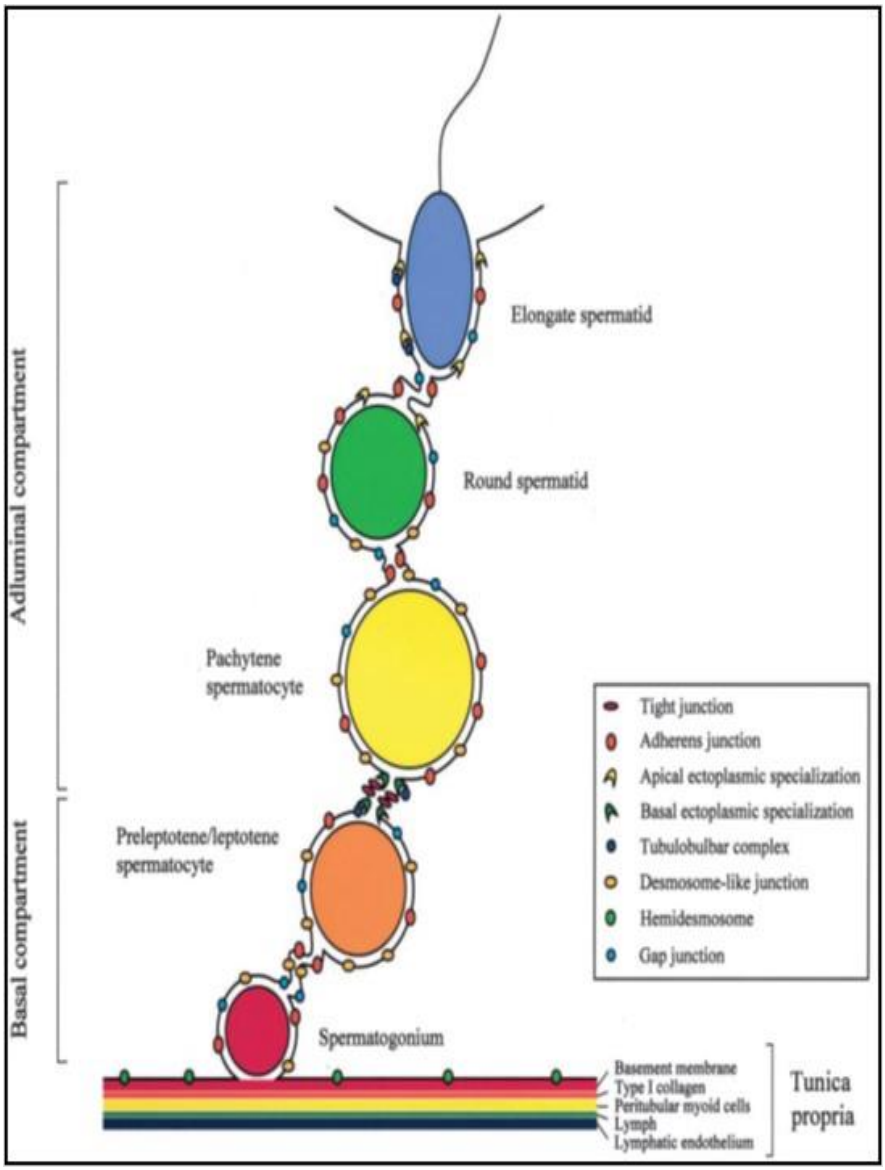
Bone Morphogenetic Protein 8B (BPM8B), member of the TGF-β superfamily, is expressed in spermatogenic cells. They act as local regulators of spermatogenesis and are triggered by testosterone. *Bmp8b* gene inactivation results in loss of germ cell and infertility (Zhao et al. 1996).

Interactive regulation of spermatogenesis by Sertoli cells includes structural Support and Barrier. Sertoli cells were previously named Nurse Cells as they support and protect the developing germ cells. Apart from providing structural support, Sertoli cells also create an immunologically impermeable barrier. The seminiferous epithelial barrier, profoundly known as the BTB, is an occluding junction constituted by Sertoli cell tight

junctions. It separates the seminiferous epithelium into two distinct compartments (Mruk & Cheng 2004; see Figure 19).

Figure 19

Schematic drawing illustrating the relative locations of the different types of junctions found in the testis.



The basal compartment houses the spermatogonia, preleptotene, and leptotene spermatocytes. The adluminal compartment comprises of meiotic spermatocytes and spermatids in various stages of spermatogenesis and spermiogenesis (Russell et al. 1978). Major function of the BTB to create a specialized environment for the germ cells protecting them from immunological responses against it's the surface antigens due to the haploid genetic content which is otherwise considered as "foreign" to the body's immune system. It also allows specific molecules to pass through it.

Sertoli cell also engages in germ cell movement by translocating early meiotic spermatocytes from basal to adluminal compartment, nourishment, and spermiation. Sertoli cells serve the process of spermiation by several means. The cytoplasmic processes of Sertoli cells encapsulate the head of spermatids. Sertoli cells also help in expulsion and release of spermatid heads into the lumen (de Kretser et al. 1988).

Sertoli cells maintain the integrity of the seminiferous tubule by acting as a macrophage. It phagocytizes excess cytoplasmic residual bodies pinched off from and the seminiferous tubule by acting as a macrophage (Carr et al. 1968; Kerr et al. 1974). Sertoli cells are subjected to active pinocytosis in the adluminal compartment and undergo receptor mediated endocytosis in the basal compartment of the seminiferous epithelium (Clermont et al. 1987; Morales et al. 1991; Rong-Xi et al. 1987).

Sertoli cells are compelled to synthesize, secrete products necessary for the growth and differentiation of the germ cells. Substances secreted by Sertoli cells include proteases, protease inhibitors, hormones, energy substrates, growth factors, and paracrine

factors. Proteases and protease inhibitors participate in maintenance, growth, and development of the germ cells (Fritz et al. 1993). Secretory factors like growth, autocrine, and paracrine bind to the surface receptors evoking a cascade of signal transduction effecting cell growth and differentiation (Ohno et al. 1984). These signaling pathways play a pivotal role in spermatogenesis and germ cell movement (Georgiev et al. 1993). The nutrients secreted by the Sertoli cells are extremely necessary for the nourishment of the developing spermatocytes and spermatids that are lodged behind the BTB. These products include amino acids, carbohydrate, lipids, vitamins, and essential metal ions (Bardin et al. 1988). Transfers of these materials are possible due to the spatial arrangement of the Sertoli with the germ cells.

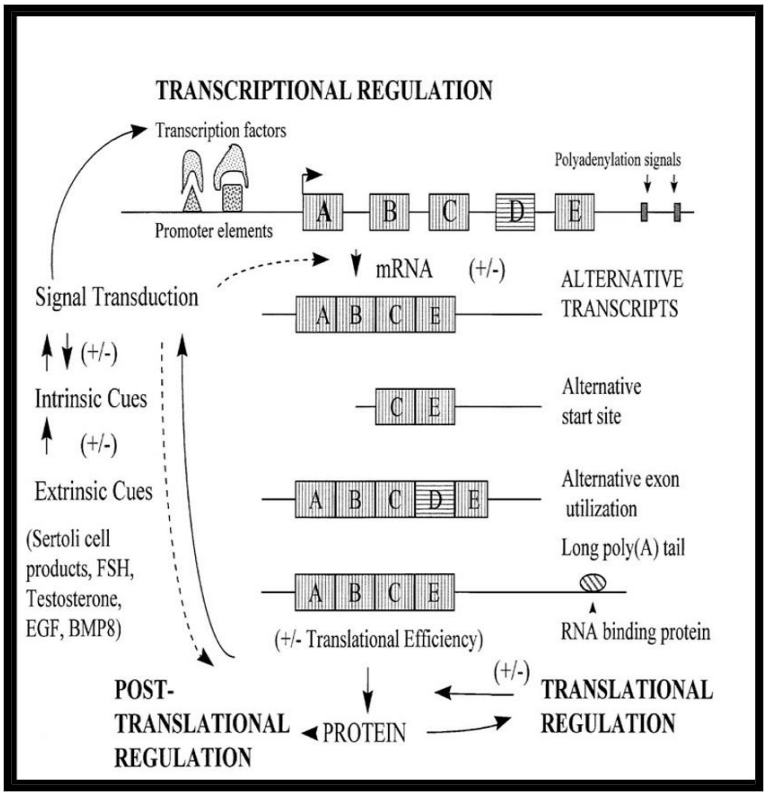
The basement membrane of testis is composed of laminin, type IV collagen, heparin sulfate proteoglycan, and entactin (Dym et al. 1994). Sertoli cells contribute to the structural integrity of the seminiferous tubule by secreting extracellular matrix like collagen (Skinner et al. 1985) and laminin (Davis et al. 1990; Ray Choudhury et al. 1992; Richardson et al. 1995).

Hormones such as testosterone (Kim et al. 2001), dihydrotestosterone (DHT), estradiol (Pentikinen et al. 2000), and cytokines like TNF-α (Pentikinen et al. 2000) also influence the function of Sertoli cells towards the germ cells. To maintain germ cell survival, Sertoli cells efficiently metabolize glucose into lactate (preferred energy source for germ cells) and pyruvate (Grootegoed et al. 1986; Robinson et al. 1981).

Intrinsic regulation of spermatogenetic gene expression occurs in four levels: transcriptional, post-transcription, translation and post-translational. Just like any other cell in the body, transcription acts as the first level determinant of gene expression. Post-transcription determines production of alternative forms of gene homologues specifically for the germ cells. Third level determinant, translation, is the most crucial point in germ cell development-specifically in post-meiotic phase. The last level of regulation occurs after the peptides are formed. Depending on the internal and external signals, the post-translational regulation differs (Eddy 1998; see Figure 20).

Figure 20

Regulation of gene expression during spermatogenesis



Transcriptional regulation occurs at the upstream TF binding motif of the target gene. Any transcription factors binding to the promoter region can be regulated in order to maintain transcription of the gene of interest and producing a functional product. Alterations can also be induced in chromatin structure thereby changing transcriptional activity. Many universally located transcription factors function in different tissues but some unique ones are restricted to tissues for specific gene regulation. Unique spermatogenic cell specific transcription factors such as SPRM1, TAK-1, ZFY-2, and OCT-2 have been identified but data determining the genes they regulate has not been found (Winer et al. 1993). Roles of cAMP responsive element binding protein (CREB) and cAMP responsive element modulator (CREM) transcription factors in spermatogenic cells have been one of the best studied examples of transcriptional regulation in spermatogenesis. Both CREB and CREM are activated by cAMP-regulated protein kinase A signaling. Alternate forms of CREB and CREM act in different phases of spermatogenesis and regulate different gene transcription by activating or suppressing CRE binding.

Presence of transcriptional regulating genes present in spermatogenic cells have been identified by foot printing assays. They reside in the nucleus of the developing spermatogenic cells and regulate by interacting with DNA promoter sequence motifs (Eddy 1998). Histone H1t TE element binding proteins (vanWert et al. 1996) and Kinase 2 promoter binding protein are two such examples of transcription factors present only in spermatogenic cells (Gebara et al. 1992).

Likewise, there are other somatic cell specific transcription factors located in the nucleus of only somatic cells but not germ cells. A well-studied examples of one such transcription factor is negative regulatory element (NRE) which binds the ***c-mos*** promoter to repress gene expression in somatic cells but absence of NRE in spermatogenic cells facilitates the expression of genes such as Protamine 2, Phosphoglycerate kinase 2, Cyt Cτ, and Hsp70. Expression of NRE may be a mode to suppress these genes in somatic cells (Xu et al. 1995).

Splicing is one of the most important mechanisms involved in post-transcriptional regulation of spermatogenesis. It can produce alternate spliced products of mRNAs to modulate expression of somatic gene homologues. Common ones found in spermatogenic cells are c-Mos, tACE, CytCτ, and α-Abl. 5' UTR of the gene transcripts are brought about by methylation and pseudouridination (Eddy 2002). Alternate polyadenylation process helps in modifying the 3' end of the transcript (Liu et al. 2006). Three variants of CD-MRP have been reported with different sizes of poly (A) tail as those tails were added at alternate sites (Hecht et al. 1998). Differential extension of poly(A) tail in Prm1 is done by cytoplasmic polyadenylation element binding protein (CPEB; Mendez et al. 2001) and testis specific cytoplasmic poly(A) polymerase (Tpap; Kashiwabara et al. 2002).

The phenotype of the germ cell is determined by the translation machinery of that cell. Germ cells exhibit exclusive structural and functional proteins and differ in its content of novel transcripts in various stages of differentiation. During nuclear

condensation occurring in Steps IX-XII, histone proteins are replaced by transitional proteins and further by protamines. In order to cater this, these genes are transcribed and stored in Ribonucleoprotein particles (RNPs; Cho et al. 2005). During the time of the specific step, specified mRNAs are released from RNPs to undergo translation. This phenomenon prevents accumulation of excess unnecessary proteins in the polysomal complex. During spermiogenesis Prm1, Prm2, Odf2, Spata-6, and Tex-27 genes are translationally upregulated to deliver them to polysomes (Cho et al. 2005).

Translational regulation of stage specific genes is brought about by associating mRNA binding proteins to their transcripts. Glutamine synthase (Glul), App, cyclin B1, and cleavage stimulatory factor 2 (Cstf2) are the genes that are not required during the late stages of spermiogenesis and are regulated by mRNA binding proteins. 40Kd Protamine 1 RNA binding protein (PRBP) binds to the 3' UTR of its target genes not required in spermatid stage (Lee et al. 1996). A large array of RNA binding proteins like TB-RBP, RBM, ATP dependent RNA helicase, DAZ, and SOD1 associates with the gene transcripts of unique genes present in the germ cells (Shima et al. 2004).

Post-translational modifications pose another level of regulation involving phosphorylation, ubiquitination and SUMOylation. Cyclins, the main regulators for cell cycle checkpoint regulation, need to be degraded at various phases of cell cycle. The process by which most proteins are degraded is mediated by poly-ubiquitin-proteolysis. Degradation signals activate ubiquitin activating enzymes E1 that transfers ubiquitin to the next enzyme E2. E2 along with E3 having a substrate recognition site assembles a

poly-ubiquitin chain on specific phosphorylated site on the substrate (Pollard et al. 2007). Cyclins during various cell cycle phases and anaphase promoting complex (APC) are degraded by ubiquitin-mediated proteolysis to release the sperm cell from metaphase checkpoint.

SUMOylation is antagonistic of ubiquitination and increases the lifetime expectancy of protein. Transcriptional, post-transcriptional, translational, and post-translational regulation is moderated via SUMOylation by changing localization and distribution of proteins (Bossis et al. 2006a; Bossis et al. 2006b).

Cells, the basic biological unit actively participating in all their functions, maintain their structure and functions by accommodating to the changing demands of extracellular stresses. Cells normally try to maintain their normal homeostasis. If cells undergo physiological stress or any external or internal stimuli, they can undergo major adaptations in order to maintain the steady state and conserve their functions. Adaptive responses include hypertrophy, hyperplasia, atrophy, and metaplasia. If the external stress exceeds the adaptive capability, the cell develops injury. Within certain limits this injury is reversible, and the cell comes back to its normal function. But in several cases the stress level produces irreversible injury causing cell death.

Cell death is one of the most important events in normal evolution of a cell. It can be caused due to diverse reasons including toxins, infections, and immune reactions. In the process of embryogenesis, development of organs and maintenance cell death is a normal and essential process.

Apoptosis involves an orchestrated series of cellular events leading to distinct form of cell death featuring DNA laddering, chromatin condensation, and membrane blebbing. In adults, spermatogenesis features distinct steps: mitotic proliferation of spermatogonia, meiotic division of spermatocytes and differentiation of spermatids, germ cell maturation, and finally release of spermatozoa into the tubular lumen.

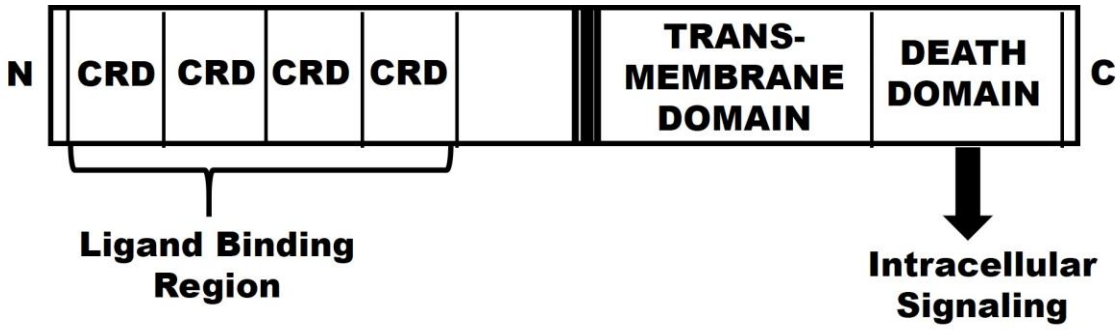
Apoptosis is accompanied by defined morphological changes that can affect either single cell or a group of cells. Initial modifications in apoptotic cells include loss of cell junctions. Lipids present on the surface of the cells are rearranged to expose phosphatidylserine towards the external face. The cell size shrinks with distinct membrane blebbing or ruffling. Inside the cell, the cytoplasm condenses markedly due to loss of intracellular fluids and ions (Schwartzman et al. 1993). Endoplasmic reticulum dilates and forms membrane bound vesicles. In the nucleus, chromatin condensation and breakdown of DNA is accompanied by nuclear fragmentation (Cotter et al. 2003). The cell changes into a convoluted structure and breaks up into membrane bound apoptotic bodies containing organelles and nuclear fragment. These vesicles are phagocytosed by normal epithelial cells, vascular endothelium, and/or mononuclear phagocyte system (Goldsby et al. 2000). Apoptotic cells in testis are phagocytosed by Sertoli cells.

Apoptosis, an actively enzymatic process involves breakdown of nucleo-proteins followed by cellular fragmentations. Activation of such enzymes is based on death domain receptors (DDRs). This class of membrane receptors is a sub-group of the TNF receptor family. Among many others, these death domain families include DR3, TNF-R1,

Fas (CD95), and TRAIL-R2. All share common structural feature: an extracellular N-terminal ligand binding region, a transmembrane region, and an intracellular C-terminal region (Hancock 2000; see Figure 21).

Figure 21

The domain structure of a typical death receptor

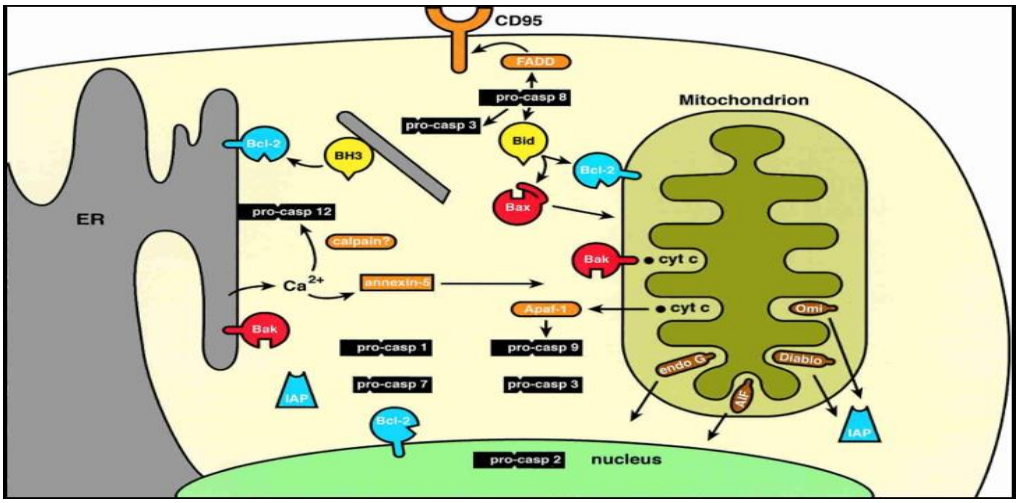


The extracellular region contains up to six cysteine rich domain (CRDs) whereas the intracellular region has a death domain involved in intracellular signaling. Ligands of these receptors form trimers and majorly consist of soluble membrane proteins. Ligand binding leads to trimerization of the receptor allowing induction of the signaling cascade (Mousavi et al. 2008). The apoptotic enzymes, specially named as CASPASES, cysteine proteases that cleave proteins after aspartic residues. All caspases share a common peptide sequence in their active site: -*Glu-Ala-Cys-X-Gly*- where *X* is any amino acid. Out of the 14 mammalian caspases, 11 are found in humans (Lodish et al. 2003). The family of caspases is divided into two main classes. The first class of caspases includes initiator or apical caspases (Caspase-2, -8, -9 and -10). Activation of these initiator caspases takes place by autocatalytic cleavages and interaction with specific adaptor

protein complexes. Caspase-2 ligates with PIDDosome complex containing an adaptor protein RAIDD and a death domain protein induced by p53. The preferred target sequence for caspase-2 is –*Val-Asp-Val-Ala-Asp-*. Caspase-8 becomes associated with DISC containing an adaptor protein FADD. In turn, caspase-9 couples up with Apoptosome, a multimeric protein containing Apaf-1, Cytochrome C, and ATP as a co-factor (Bao 2007). The preferred target sequence for caspase-8 and -9 is – *(Ile/ Lev/Val)-Glu-X-Asp-*. A general characteristic of all these caspase adaptor proteins is the presence of caspase recruitment domains (CARD) at its amino terminal end. The second class of the caspase family, effector or executioner caspases, includes caspase-3, -6, and-7 (Rield et al. 2004). These are activated by cleaving their pro-forms with the initiator caspases-8 or-9 to release their active sub-units (Kumar et al. 2007; Van Loo et al. 2002; see Figure 22).

Figure 22

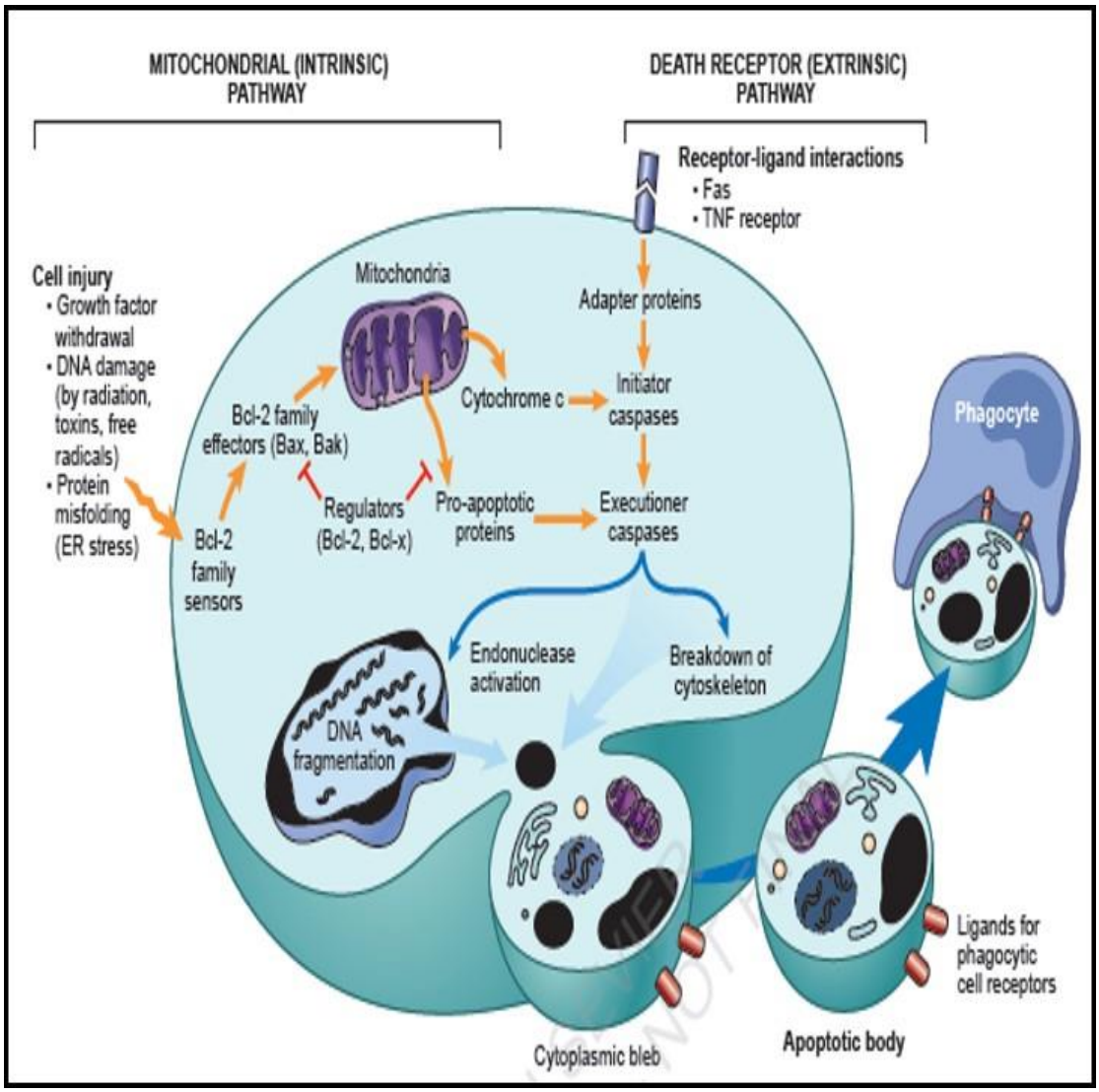
The location of caspases and their major regulators



The preferred target sequence for caspase-3 and-7 is *–Asp-Glu-X-Asp-*. This chain of caspase activation is initiated and regulated by death signals received from two distinct pathways (Ravichandran 2003; see Figure 23): (i) The death receptor or extrinsic pathway, (ii) the mitochondrial or intrinsic pathway.

Figure 23

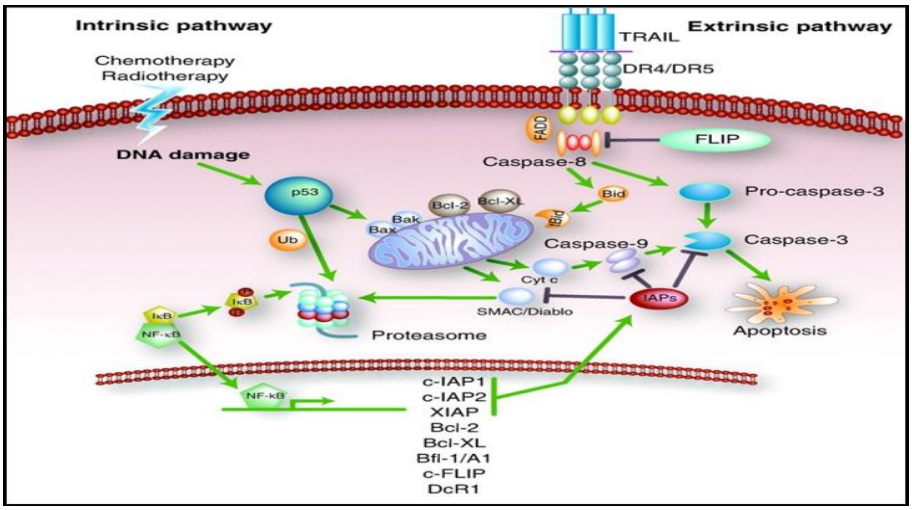
Pathways to apoptosis



Extrinsic, as the name suggests, it involves external stimulation to induce an orchestrated signaling pathway for cell death. Fas L, a membrane protein or any other extracellular ligand interacts Fas, the death domain receptor, to start the extrinsic pathway. As the receptor receives the signal, it trimerizes to activate itself and binds to an intracellular protein FADD via its death domain. Upon binding, FADD associates with procaspase-8 to initiate its self-splicing and producing active caspase-8. As the initiator caspase, caspase-8 is activated; it cleaves off procaspase 3 and 7 into caspase-3 and-7 respectively. Caspase-8 also cleaves and activates Bid, a pro-apoptotic member of the Bcl2 family. Bid then acts as one of the co-regulators of the mitochondrial or intrinsic pathway. Caspase-3 and-7 in turn initiates the caspase cascade to result in controlled proteolysis to carry out cell's suicidal process (Layrik et al. 2005; see Figure 24).

Figure 24

Extrinsic and intrinsic pathways of apoptosis

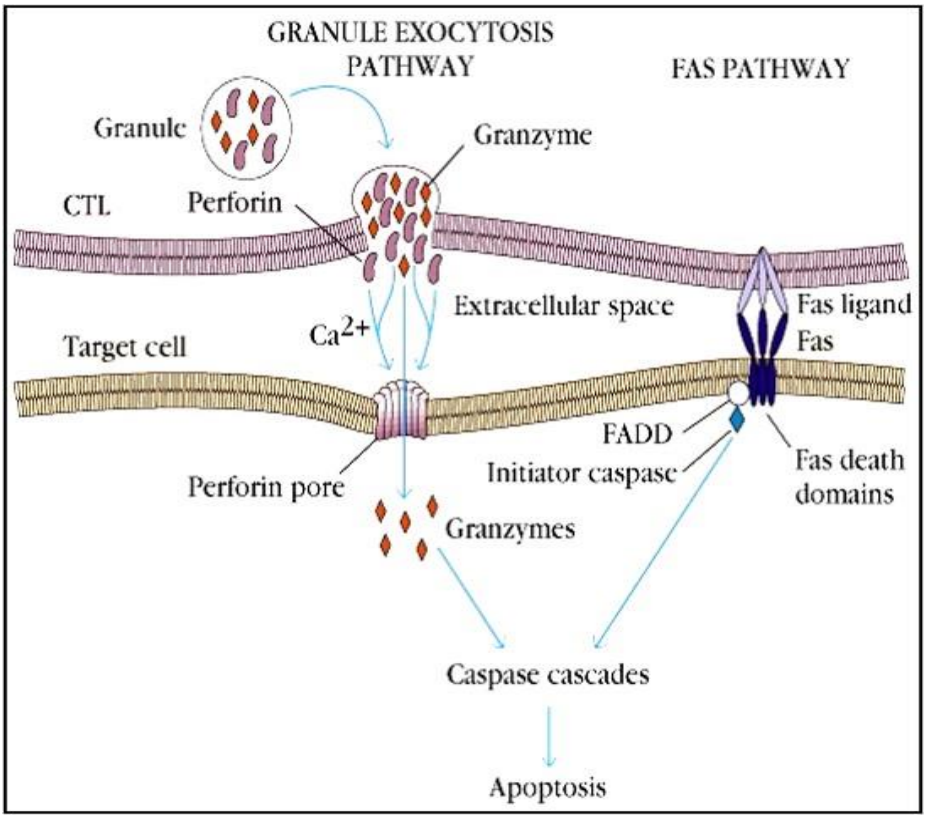


Note. CCR Molecular Pathways.

After receiving death signal, the storage granules containing perforins and granzymes are coupled with ligand receptor interaction. The contents of the storage granules are released into the junctional spaces of the cells. Perforins from these granules bind with the calcium ions that are present forming pores on the membrane of the target cells (Lu et al. 2006). Granzyme, a serine protease, enter through these pores to activate effector caspases to initiate apoptosis (Goldsby et al. 2000; see Figure 25).

Figure 25

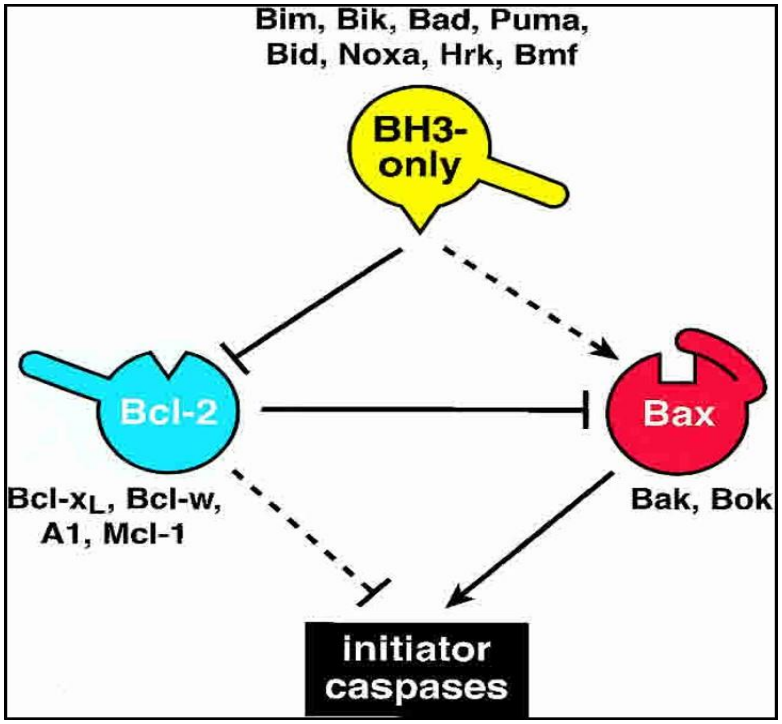
Proposed model for initiation of apoptosis by granzyme/perforin mediated or Fas/Fasl induction.



Mitochondria play an important role in the process of apoptosis. It contains several proteins capable of inducing apoptosis. These include cytochrome C and other antagonists for apoptosis inhibitors. Cell viability is thus determined by the permeability of mitochondria (Chipuk et al. 2006). This permeability is controlled by a family of proteins, Bcl2 family. This family of protein involves 20 proteins divided into three major groups based on their Bcl2 homology and function: (a) Multi domain pro-apoptotic proteins (Bax and Bak); (b) BH3 only pro-apoptotic proteins (Bim, Bid, and Bad); and (c) Multi domain anti-apoptotic proteins (Bcl2 and Bcl-X_L; Certo et al. 2006; Cuconati and White 2002; see Figure 26).

Figure 26

The three major mammalian factions of the Bcl-2 family

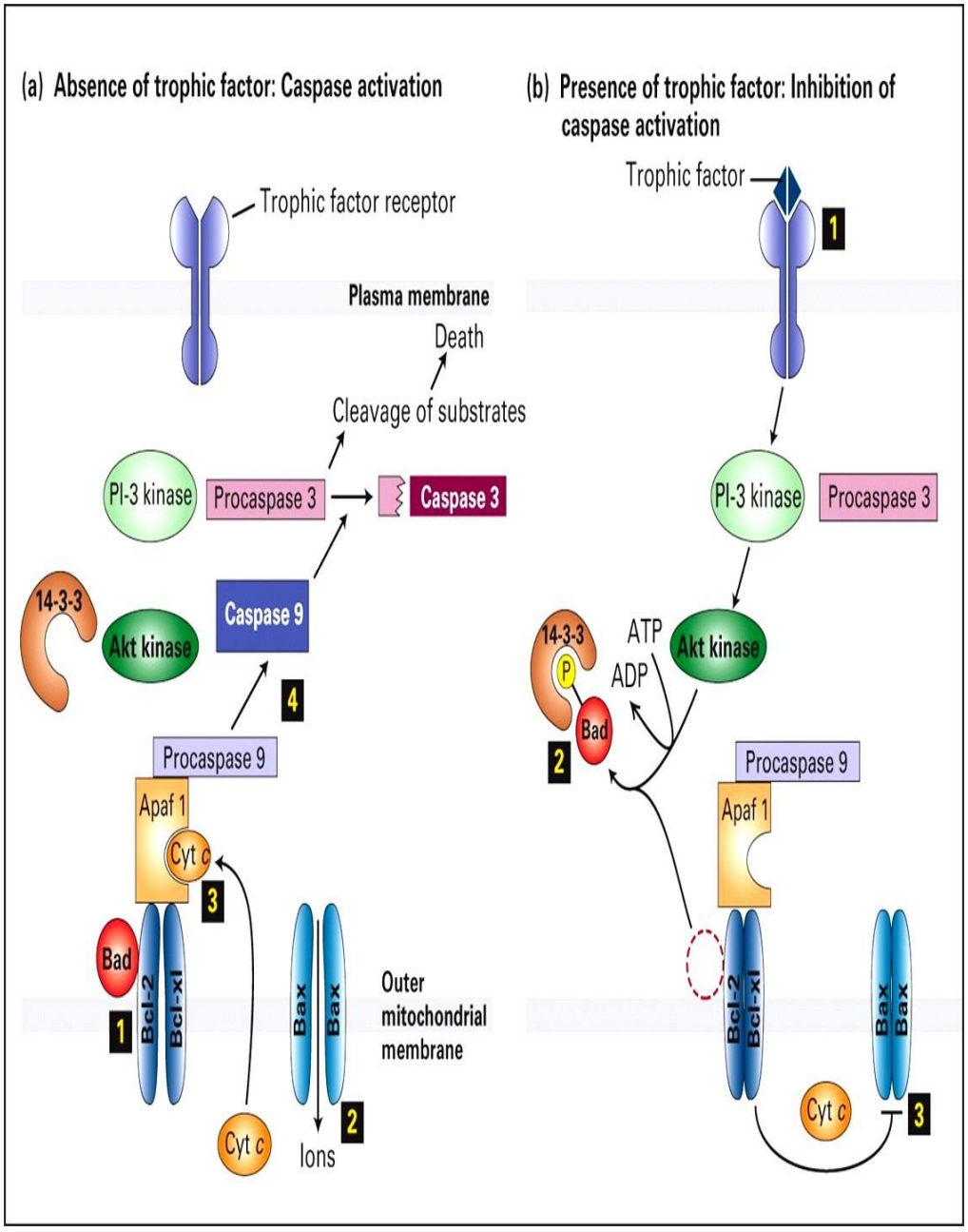


Cells deprived of growth factors and trophic hormones, exposed to DNA damaging agents and/or have accumulation of misfolded proteins (Kaufman et al. 2002), stimulate a series of sensors dephosphorylating soluble pro-apoptotic protein, Bad. Upon activation, Bad release itself from the 14-3-3 complex and bind and inactivate the cytosolic heterodimer of Bcl2-Bcl X_L. Thus this complex is incapable of suppressing Bax. Activated Bax undergoes conformational change and translocates itself to the mitochondrial outer membrane with the help of novel Bax chaperone, nucleophosmin (Kerr et al. 2007). Bax homo-oligomerize to form channel in the outer membrane of mitochondria to mediate influx of cytoplasmic ions. As a result, mitochondria are induced to release cytochrome C which binds with Apaf-1 in the cytoplasm to form apoptosome complex (see Figure 24). This complex cleaves procaspase-9 to from activated caspase-9. Caspase-3 is proteolytically cleaved from its pro-form by activated caspase-9 to initiate cell death (Lodish et al. 2003; see Figure 27-a).

During normal cellular condition, Akt kinase phosphorylates Bad to bind it with the 14-3-3 and inactivate it. In the absence of Bad, Bcl2, and Bcl-X_L heterodimer interacts with Bax and inhibits Bax to form oligomerized pore, thus maintaining the permeability of mitochondria (Lodish et al. 2003; see Figure 27-b).

Figure 27

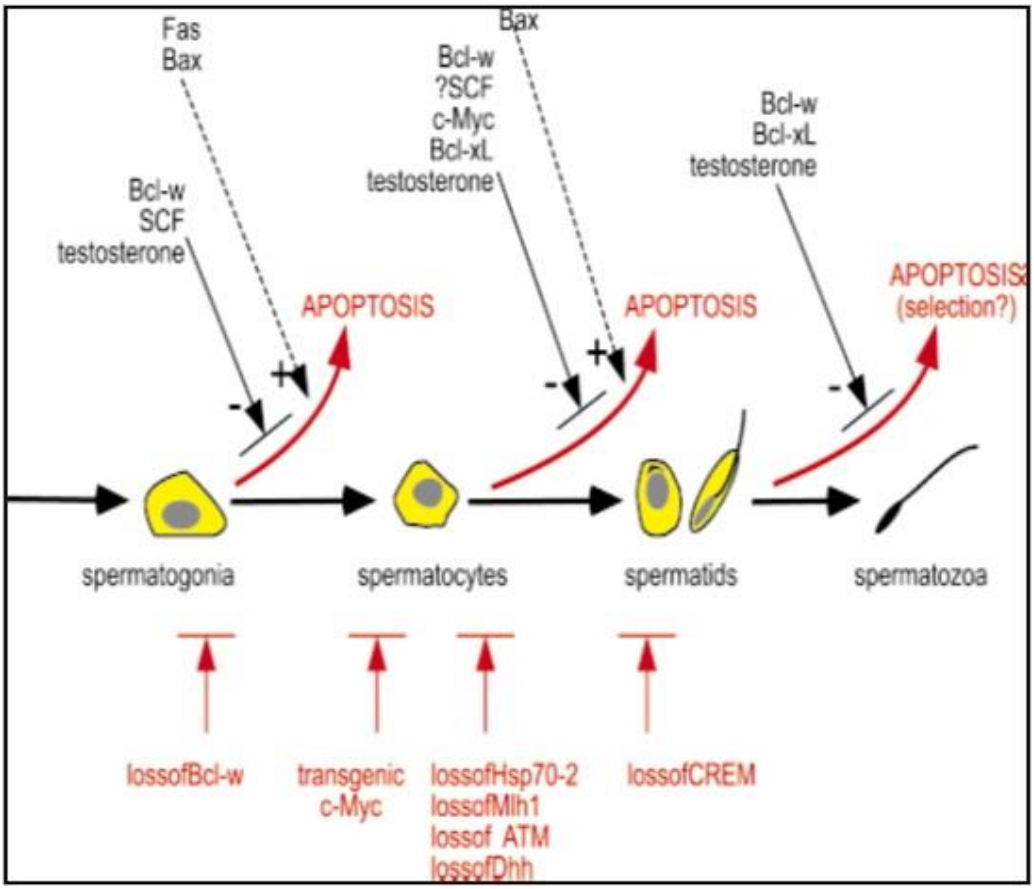
Caspase activation and inhibition (a) Caspase activation in the absence of trophic factors; (b) Inhibition of caspase action during the presence of trophic factors.



Germ cell apoptosis is noticed as early as at the first wave of spermatogenesis. Mouse studies by Rodriguez et al. (1997) and Wang et al. (1998) showed that apoptosis in germ cells during the first wave of spermatogenesis can be identified at its peak at 2 weeks after birth. This is supposed to be the first step in the maintenance of Germ cell to Sertoli cell ratio and was coupled with multiple signals (Orth et al. 1988; Print & Loveland 2000; Rodriguez et al. 1997; Wang et al. 1998; see Figure 28).

Figure 28

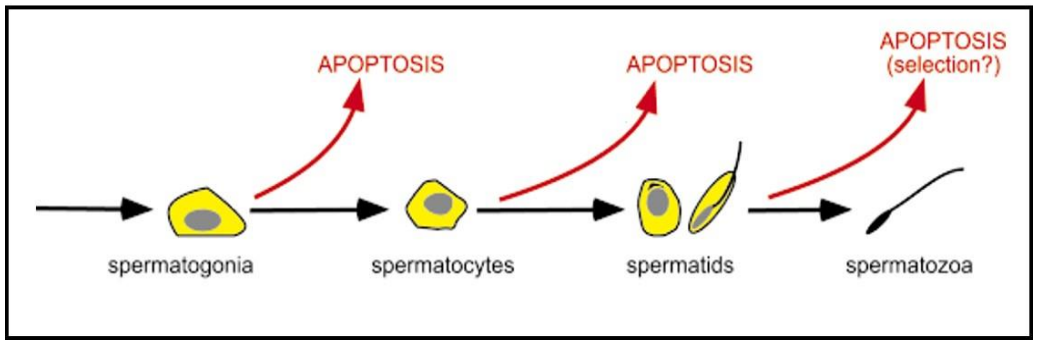
Germ cell apoptosis maintains appropriate numbers of each germ cell type, and selectively removes damaged cells



A regulated developmental sequence involving germ cell proliferation, self-renewal and apoptosis is the route towards the successful process of spermatogenesis where 75% of the germ cells produced are discarded through apoptosis (Shaha et al. 2010). A balance of the paracrine signaling from stem cell factor (SCF), leukemia inhibitory factor (LIF), and desert hedgehog (DHH) in addition to endocrine signaling from pituitary gonadotropins and testosterone mediate germ cell survival and death in the seminiferous tubule. Apoptotic germ cells lose cell to cell junction and are sloughed off into the lumen where they are engulfed by Sertoli cells (Print & Loveland 2000; see Figure 29).

Figure 29

Germ cell apoptosis



Apoptosis in spermatocytes and elongated spermatids is unclear as they do not have any characteristic nuclear changes perhaps because of their uncommon DNA configuration and chromatin morphology. The residual bodies can be removed either by Sertoli cells or by nuclear-independent apoptotic processes. As mature spermatozoa do

not contain much cytoplasm, classical apoptotic process involving cytoplasmic regulators may not take place (Allan et al. 1987).

In adult mammal testes, 75% of the spermatozoa are degenerated (Allan et al. 1987; Huckins et al. 1978). This result confirms that germ cell apoptosis is just a portion of the considerable loss of germ cells during spermatogenesis. But the exact proportion or percentage of apoptotic germ cells within the total pool of degenerating germ cells remain unclear as not every degenerating cells share morphological similarities with apoptotic cells. As degenerating spermatogonia and round spermatids show morphological and biochemical features like the apoptotic cells, it can be said that the majority or total of those cell population die due to apoptosis. Regression of round spermatids often features multinucleate symplasts due to incomplete cytokinesis. This acts as a distinguished feature in spermatogenesis. Apoptotic germ cells lose cell to cell junction and are sloughed off into the lumen where they are engulfed by Sertoli cells. Apoptosis in spermatocytes and elongated spermatids is unclear as they do not have any characteristic nuclear changes perhaps because of their uncommon DNA configuration and chromatin morphology. The residual bodies can be removed either by Sertoli cells or by nuclear-independent apoptotic processes. As mature spermatozoa do not contain much cytoplasm, classical apoptotic process involving cytoplasmic regulators may not take place (Weil et al. 1998).

During embryonic germ cell development paracrine signals serve as major regulators of germ cell apoptosis. In vitro studies performed by Olaso (1998) showed a

role of TGF-β in gonocyte apoptosis (Olaso et al. 1998). The Fas system is identified as one of the major paracrine signaling system in testes. Germ cell apoptosis is mediated by activation of Fas and FasL. FasL expressed on Sertoli cells bind to Fas which are expressed on germ cells to activate the cytoplasmic death domain of Fas, FADD which in turn promote a series of IL-1b converting enzyme family of protease activity. As the downstream effector caspases are activated, they cleave various cytoplasmic and nuclear components like lamin, fodrin, actin, PARP-1 and -2, Granzyme N (Takano et al. 2004), Granzyme K, and DNA dependent protein kinase; all of these resulting in apoptosis.

Gonadotrophins (FSH and LH) as well as testosterone influence germ cell fate (Tapanainen et al. 1993). Removal of these hormones induces germ cell apoptosis probably due to indirect effects as the receptors for these hormones are present on Sertoli cells but not on germ cells (Billig et al. 1995; Nandi et al. 1999; Russell et al. 1977; Woolveridge et al. 1999). This report was further validated by increased apoptosis in all germ cells when they were administered with oestradiol (Blanco-Rodriguez et al. 1997). hCG increased spermatogonial apoptosis and resulted in smaller testis volume and lower sperm count in humans (Dunkel et al. 1997). Hormone withdrawal treatment affects gene expression of Bcl2 family of proteins.

Apoptosis in spermatogonial cells is regulated by expression and suppression of apoptotic genes. Anti-apoptotic genes Bcl2 and Bcl-X_L **(**Beumer et al. 2000) are the best studied examples of apoptotic genes along with the other pro-apoptotic members of the Bcl2 family (Bax, Bak, Bcl-X_S, and Bad; Rosenwald et al. 2001). In this respect, one of

the most critical determinants of germ cell fate is the ratio of pro- and anti-apoptotic protein expression. Excess of pro-apoptotic protein led to cell death whereas excess anti-apoptotic proteins result in cell survival. Abundant expression of Bax during 1 to 3 weeks after birth proves that it is important for inducing apoptosis in spermatogonia and spermatocytes to maintain normal germ cell homeostasis during the first wave of spermatogenesis (Rodriguez et al. 1997).

Spermatocytes which are unable to carry out the complete process of meiosis are selectively eliminated by apoptosis. Mouse spermatocytes with unpaired chromosome are potential targets for apoptosis (Odorisio et al. 1998). Mice spermatocytes lacking Hsp 70.2 (Dix et al. 1996) and DNA mismatch repair protein Mlh-1 (Baker et al. 1996) cannot go through meiosis and are readily eliminated by apoptosis. Studies show a high rate of spermatocytes and round spermatid apoptosis in infertile male (Tesarik et al. 1998).

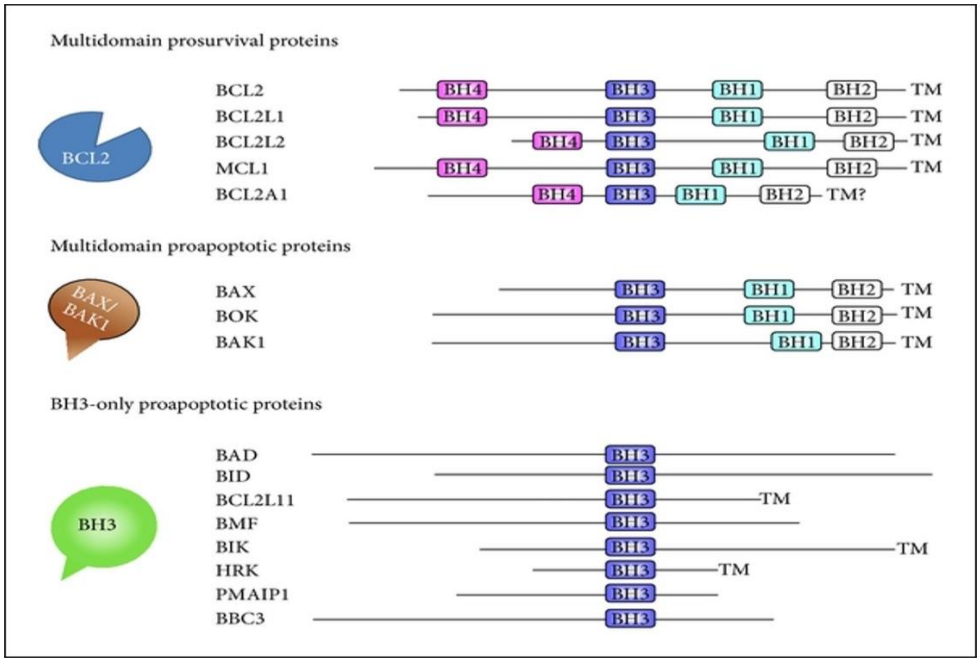
Spermatogonia damaged by ionizing radiations (Allan et al. 1987) and haploid spermatids lacking CREM protein (transcription factor for post meiotic germ cell specific genes) are eliminated by selective apoptosis (Nantel et al. 1996). Further understanding of the apoptotic pathways is necessary to develop strategies for rescuing stress induced germ cells.

Bcl-2 Homology (BH) domains are found in all proteins belonging to the Bcl-2 family. There are four distinct BH domains - BH1, BH2, BH3, and BH4. Anti-apoptotic Bcl-2 family proteins (including Bcl-2, Bcl-XL and Bcl-XW) share BH1 and BH2 domains and in some cases, a BH4 domain. Most pro-apoptotic family members, such as

Bax and Bak, contain a BH3 domain, which may also be present in some anti-apoptotic proteins as well (i.e., Bcl-2 and Bcl-xL). BH domain proteins control mitochondrial-induced apoptosis by either promoting or preventing cytochrome c release into the cytosol and the subsequent regulation of caspase-9 activity. The BH3 domain promotes dimerization of Bcl-2 family members. Homodimerization of Bcl-2 involves a head-to-tail interaction in which the N-terminal region, containing the BH4 domain, interacts with the more distal region of Bcl-2 where BH1, BH2 and BH3 are located. Conversely, Bcl-2/Bax heterodimerization involves a tail-to-tail interaction that requires the BH1, BH2, and BH3 domains of Bcl-2 and a central region of Bax that contains a BH3 domain (Olsson et al. 2013; Sattle et al. 1997; see Figure 30).

Figure 30

Classification of the BCL2 family into three subfamilies of BCL2-related proteins



Apoptosis leading to germ cell death varies according to the cell types in the seminiferous tubules. Different germ cells produce unique proteins during their development that enables them to be identified. Undifferentiated spermatogonial cells commonly express surface protein markers α-6-integrin (Shinohara et al. 1999) and CD9 (tetraspin transmembrane protein; Ito et al. 2010). Prohibitin is a mitochondrial protein involved in DNA damage repair. They are expressed constitutively in Leydig cells and Sertoli cells at all stages and at a very low level in preleptotene spermatocytes. Prohibitin protein is at a very high level in leptotene spermatocytes and at a low level in zygotene spermatocytes. In pachytene spermatocytes, Prohibitin is expressed at a very high level in stages VII – XI and minimal during stages XII and XIV of the spermatogonial wave (Choongkittaworn et al. 1993). Spermatogenesis-related factor (SRF-1) gene product is a marker involved in meiotic spermatocytes. It was detected at high level in leptotene and pachytene spermatocytes (Yamano et al. 2001). Boule is a member of DAZ gene family that encodes protein that is distinct from other RNA binding proteins. It is expressed in the cytoplasm of pachytene spermatocytes, persists through meiosis and decreases in early spermatids (VanGompel et al. 2010). Transition protein 1 (TP1) are proteins that are involved in the packaging of sperm nuclear DNA during spermiogenesis. They take the place of histones associated with the sperm DNA and are subsequently themselves replaced by protamines. Round spermatids produce and store mRNAs for transition protein and protamine and express TP1 as its unique protein marker (Sassone-Corsi et al. 2002). Lactate dehydrogenase (LDH) participates in anaerobic glycolysis. LDH-C4 is a

testis specific lactate dehydrogenase uniquely suited for satisfying the metabolic requirements of differentiating germ cells and functional spermatozoa. LDH-C4 gene is expressed exclusively during meiosis and spermiogenesis, beginning in leptotene/zygotene spermatocytes and continuing through the elongated spermatids (Odet et al. 2008). Protamine 1 is involved in histone synthesis during spermiogenesis. The histones are replaced by the set of transition proteins that are subsequently replaced by protamines. Protamines gene is transcribed post meiotically in the round spermatid stage of spermatogenesis and is used to predict the presence of testicular spermatozoa (Steger et al. 2001). Acrosin, associated with the acrosome of the spermatozoa, is a post-meiotic marker that is involved in compaction of chromosomal matrix (Kashiwabara et al. 1990; Klemm et al. 1991). Sperm protein-10 (SP-10) is a mouse sperm acrosomal protein associated with the outer and inner acrosomal membrane of the anterior acrosome and present within the equatorial segment. SP-10 is transcribed post meiotically in the round spermatid and spermatozoa stages of germ cell development. cAMP responsive element modulator (CREM) is involved in regulating gene expression in haploid spermatids. CREM transcriptional activity is controlled through the interaction with testicular compartment cells. This is highly specific for post meiotic male cells where it regulated number of genes involved in spermiogenesis (Weinbauer et al. 1998).

While it is known that apoptosis of meiotic cells is high with the loss of testosterone and these cells are exfoliated from Sertoli cells, the direct effect of testosterone loss upon germ cells can never be determined. If this loss of testosterone is

vital for the loss of germ cell functionality, it can also disrupt the cytoskeleton in testis. Microtubules and microfilaments are involved in multiple aspects of Sertoli and germ cell development, and in many cases the structures formed are analogous to those in other parts of the body. The Sertoli cell cytoskeleton contains abundant microtubules. The microtubules are oriented in linear arrays parallel to the long axis of the cell, from the base to the apex, forming a longitudinally oriented cage-like structure around the nucleus (Vogl et al. 1995). Microtubules are not readily apparent in regions of Sertoli cells adjacent to spermatogonia and spermatocytes but are evident in the lateral processes surrounding round spermatids and become particularly abundant in processes surrounding elongating spermatids (Amlani & Vogl 1988). Sertoli cells show dynamic changes in their microtubule patterns as germ cells progress through the various spermatogenic stages (Vogl et al. 2009). Although microtubules in the spermatogonial cytoplasm are labelled by antibodies to α-tubulin, these microtubules do not appear to be extensively post-translationally modified (Fouquet 1994). Spermatogonia nucleate their microtubules from the centrosome (Vogl et al. 1995). Spermatogonial spindles are oriented perpendicular to the basement membrane thus allowing oriented cell division. This process is likely regulated by forces exerted by astral microtubules (Lagos-Cabré et al. 2008); however, its purpose is unclear. During prometaphase, spindle microtubules are nucleated and attach to the kinetochores and spindle poles as the metaphase spindle is assembled. Various meiosis spindle checkpoints must be satisfied prior to entry into metaphase to ensure correct chromosome de-synapsis and spindle attachment (Eaker et

al. 2002; Hamer et al. 2008; Odorisio et al. 1998). During metaphase and anaphase I, the meiotic spindle moves the homologous chromosomes, each consisting of a pair of sister chromatids, towards the spindle poles, prior to the initiation of telophase and then cytokinesis. This division produces two secondary spermatocytes that rapidly enter meiosis II, whereby the sister chromatids are separated to finally produce four haploid round spermatids. However in both Sertoli cells and germ cells, questions remain regarding the sites of microtubule nucleation and the mechanisms governing their control. It is clear that the regulation of microtubule dynamics is essential for male fertility, based on the number of mouse models exhibiting abnormal sperm number and/or function (O'Donnell, & O'Bryan, 2014). Thus, spermatogenesis is an excellent model in which to study microtubule dynamics and the assembly of microtubule-based structures.

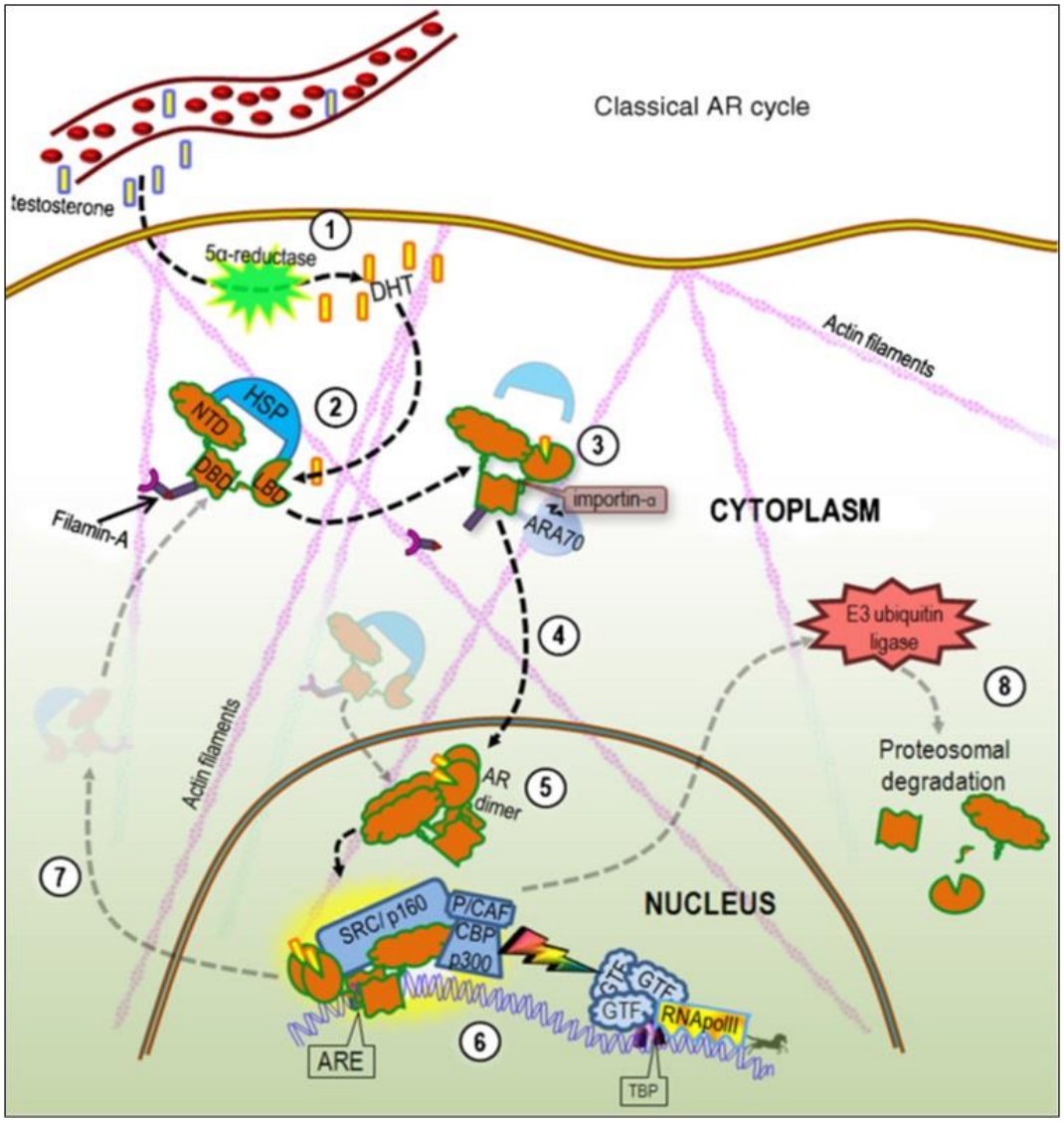
Study of the cycle of the seminiferous epithelium requires precise definition of the stages. The classification of the stages of the cycle is based on the changes observed in the nucleus and acrosomic system of the spermatid as it transforms into a spermatozoon, a process divided into 19 steps. The first 14 steps, which have been used to subdivide the cycle into 14 corresponding stages, can be used to identify the differences. The carbohydrate containing substance which is present in the acrosomic system of the spermatid is well preserved by some preservatives but poorly preserved and dissolved away by many others (Dutta et al 2012). Since the definition of the stages is based on the shape of well-fixed acrosome and head cap and precursors, it is crucial to understand the stages according to changes in testosterone concentration in the body. It can be important

to understand any changes with respect to testosterone which can be crucial for male infertility studies.

Testicular T is very high compared to serum levels and manifests action in tissues that occur much more rapidly than would be expected through the canonical pathway of activated nuclear receptors changing gene expression and ultimately the tissue phenotype (see Figure 31; Bennett. et al 2010). Instead, non-genomic/non-canonical actions of T may be with plasma membrane receptors or ion channels that are associated with other steroid membrane receptors located on plasma membranes of germ cells. Thus, it is possible that the initial phases of high T effects may be through other types of receptors such as membrane receptors (Falkestein et al. 2000). These actions may bind to membrane receptors with lower affinity but still activate the receptor by binding the steroid receptor by keeping the ligand binding site occupied and the receptors activated due to higher concentrations of T in the testicular tissue.

Figure 31

Classical AR cycle.



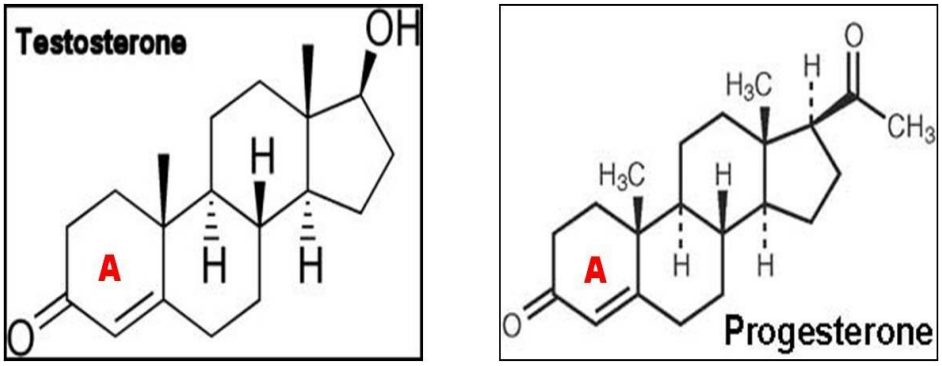
Note. Androgen receptor (AR) (colored orange) is illustrated as a modular protein with each of its domains represented; NTD (N-terminal transactivation domain), DBD (DNA-binding domain), the hinge and the LBD (ligand-binding domain). Following

the numbering in the figure: (1) Conversion of testosterone to DHT via the enzyme 5α-reductase in basal epithelial cells within the prostate; (2) DHT moves into the cytoplasm of epithelial cells where it is bound by AR; (3) Ligand binding to AR induces an AR conformational change whereby proteins such as heat shock proteins (HSPs) dissociate and others such as importin- and androgen receptor-associated protein-70 (ARA70) are recruited to help stabilize AR and **(**4) promote AR nuclear translocation; (5) AR dimerizes in the nucleus; (6) Numerous other co-activators bind to AR in the nucleus then the AR-DBD facilitates nucleic acid binding at androgen response elements (ARE) which promote recruitment of co-activators with histone acetyltransferase (HAT) activity (CBP/p300, P/CAF), via SRC/p160 co-activator family members, resulting in chromatin remodeling. This allows binding of TATA binding protein (TBP) followed by general transcription factors (GTF) and RNApolII to begin transcription; (7) Non-ligand-bound AR is shuttled back to the cytoplasm and re-cycled in preparation for further ligand binding; (8) Alternatively, non-ligand-bound AR can be targeted for proteosomal degradation following ubiquitination by E3 ubiquitin ligase.

Wenging Cao et al. (2007) demonstrated with crystallography that T binds to its receptor via the A-ring structure. Likewise, steroids such as progesterone that have an identical A-ring should have binding affinity for similar receptors (see Figure 32).

Figure 32

Structure of Testosterone & Progesterone showing similar A-rings needed for receptor binding.



Progesterone (P) has multiple reproductive functions in female, regulating reproductive activities in accessory sex tissues. In males, the expression of the genomic/nuclear progesterone receptor (PR) and physiological functions at the molecular level are not well understood.

The PRs are members of nuclear receptors subfamily 3 group C that includes: (1) aldosterone receptor (NR3C1); (2) glucocorticoid receptor (NR3C2); (3) progesterone receptor (NR3C3); and (4) androgen receptor (NR3C4). All of these receptors have strong structural similarities and binding to the 3-keto group on the A ring of steroid. The progesterone receptor is expressed in two isoforms, PR-A and PR-B. They are the product of two different transcriptional start sites and differ by an N-terminal extension

of 165 amino acids in PR-B (Conneely at al 2001; Conneely at al 2002). Mature PR-A (94 kDa) and PR-B (116 kDa) when induced by LH in granulosa cells have partly different functions. PR-A is a repressor of PR-B which is a strong activator of target genes. A shorter (60 kDa) isoform PR-C (Wei & Miner 1994) is translated from an in-frame ATG codon at the C-terminus of exon 2 of the PR gene – comprising the second zinc finger and the hormone binding domain. It is localized in the cytoplasm where P binding of this receptor allows migration into the nucleus but has no DNA binding domain, hence, does not bind directly to DNA. However, it dimerizes with PR-A and PR-B increasing their transcriptional activity (Wei et al 1996).

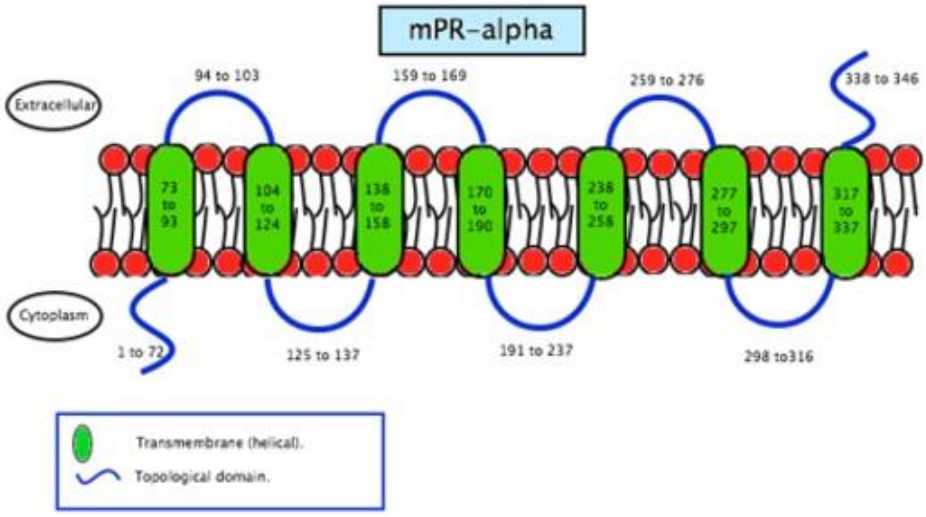
Studies of P signaling have focused on nuclear receptors but P is also known to change ion influx and intracellular Ca2+ along with other second messengers that are usually associated with signal transduction/G-protein receptors across the plasma membrane. Rapid effects of P on human spermatozoa have attracted much attention during the last few years. The sperm acrosome reactions apart from other important functions are achieved through non-genomic pathways mediated by membrane progesterone receptors (mPRs; Alexander et al 1996; Baldi et al 1995).

mPRs belong to a large class of 7 transmembrane domain receptors called progestin-adipoQ receptors (PAQRs), that include receptors for adiponectin in vertebrates. This family of membrane receptors is divided into three subgroups according to the structure and ligand specificity: (Class I) adiponectin receptors (Adipoqr1, Adipoqr2); (Class II) progesterone adiponectin receptors (PAQRs, mPRs),

PAQR7 (mPRα), PAQR8 (mPRβ), PAQR5 (mPRγ), PAQR6 (mPRδ), and PAQR9 (mPRε) (Smith et al. 2008), and (Class III) hemolysin III (Hly3r; Baida & Kuzmin 1996; Lyons et al. 2004; Tang et al. 2005; Thomas 2008; see Figure 33).

Figure 33

Seven transmembrane domains of membrane progesterone receptors



Adiponectin, a protein hormone synthesized by fat cells, is secreted into circulation but is found at reduced levels in obesity linked diseases including insulin resistance/type 2 diabetes and atherosclerosis. Adiponectin affects target tissues by binding to the plasma membrane adiponectin receptors (AdipoR1; AdipoR2; Yamauchi et al. 2014).

mPRs are comprised of at least five different receptors, which as a group have no sequence homology with nuclear steroid receptors (Nutu et al 2009). mPRs (PAQRs) exhibit seven transmembrane domains resembling G-protein coupled receptors (GPCRs)

and the mPRs seem to control G-protein associated cAMP cascades in reproductive tissues (Moussatche & Lyons 2012). Three mPRs isoforms [mPRα (PAQR7), mPRβ (PAQR8) and mPRγ (PAQR5)] were first identified in eggs of the Atlantic Croaker, a food fish (Zhu et al 2003), and subsequently in human (Thomas et al 2013; Pang et al 2013), and each mPR exhibited high affinity progesterone binding.

In the mature adult, mPRs are expressed in the reproductive tract including fallopian tubes, endometrium, myometrium and in fetal tissues activate G protein signaling in multiple tissues. mPR β and γ are both expressed in the ciliated cells lining the fallopian tubes of mice and humans. It is clear from multiple reports that mPRs are expressed in a variety of both male and female reproductive tissues. In males, mPRs appear linked to the regulation of sperm motility and these receptors may prove useful targets for male infertility treatments. While the expression of both PR and mPRs in some tissues may provide functional redundancy to ensure reproductive success via progesterone driven actions, there are many instances where the two classes of progesterone receptors clearly act independently to initiate a physiological function. Given that the binding affinities of mammalian mPRs for synthetic progestins (both agonists and antagonists) differ greatly from those of classical PRs that are co-expressed with these receptors. mPRs may present novel targets for reproductive therapies unique to mPR actions, while preserving the actions of classical PRs (Dressing et al 2011; Dressing et al 2012; Dressing et al 2014). For a long time, the identity of these receptors was uncertain, however, experimental evidence from recent years demonstrates that two types

of membrane proteins unrelated to PR (NR3C3) can mediate progesterone effects: the membrane progesterone receptors (mPRs) and the progesterone receptor membrane components (PGRMCs; Peluso et al 2014; Valadez et al 2016).

Progesterone receptor membrane component 1 (PGMRC1) and the closely related PGMRC2 belong to the membrane-associated progesterone receptor (MAPR) family are involved in P signaling in vertebrate reproductive tissues and in brain. PGMRC1 displays high affinity progesterone binding that is 2 to 10-fold greater than their affinity for T and glucocorticoids. PGMRC1 and PGMRC2 also bind/interact with mPRs. No signal transduction pathways by PGMRC1 binding of progesterone have been described to date, although motifs for tyrosine kinase, kinase binding, SH2 and SH3 have been predicted from the amino acid sequence. Evidence of antiapoptotic effects for progesterone bound PGMRC1 were shown in rat granulosa cells. As well, PGMRC1 may be an intermediary in the progesterone induction of the acrosome reaction in mammalian sperm. Additional basic information of molecular and cellular mechanisms of mPRs and PGMRC1 interactions with P and potentially T through signal transductions pathways are yet to be identified and explored (Thomas 2008). Nontraditional pathways of T action through mPRs and other novel proteins may be involved in germ cell development and thus play a significant part in germ cell maintenance and production in normal spermatogenesis.

Therefore, the focus of the project was to determine whether testosterone could bind to membrane progesterone receptors on germ cells in presence of low level of progesterone to maintain spermatogenesis.

CHAPTER II

MATERIALS AND METHODS

Chemicals and drugs

Ethylene dimethane sulfonate (EDS) is not commercially available. EDS was synthesized in our laboratory using a previously described method (Jackson & Jackson 1984). The synthesis product (EDS) was twice recrystallized and was >98% pure as assessed high performance liquid chromatography (HPLC). EDS was dissolved at 30 mg/mL in 25% DMSO + 75% H_2O and injected at 75 mg/kg body weight. Testosterone propionate (TP; Sigma) was dissolved in sesame seed oil (SSO; Sigma) at 20 mg/mL and administered subcutaneously (sc) at 10 mg/kg body weight. Solvent controls were 25% DMSO + 75% H_2O and/or SSO and were given in equivalent volumes used for EDS or T treatments.

Animals

Adult male Sprague-Dawley rats weighing 250-300 gm were purchased from Charles River Laboratories and acclimated in the University Vivarium for at least 10 days with water and food *ad libitum.* Protocol for animal care and use was followed in accord with NIH guidelines and as approved by Texas Woman's University's *Institutional Animal Care and Use Committee* (IACUC).

Thirty rats were weighed and assigned to five treatment groups with $n = 6$/group. The groups were: [1] no treatment (NT); [2] Vehicle [(25% DMSO in H_2O) + SSO]

(VEH); [3] testosterone-supplementation in SSO with DMSO+H_2O (T); [4] EDS in DMSO and H_2O + SSO (EDS); and [5] EDS in DMSO and H_2O + testosterone replacement in SSO (EDS + T). Dosages (EDS or DMSO) were given in accord to body weight intra-peritoneally (ip) on day '0' only. EDS treatment was at 75 mg/kg body weight (groups 4 and 5) and controls (groups 2 and 3) receiving 25% DMSO - H_2O at the same volume/kg body weight. Testosterone propionate in sesame seed oil was given subcutaneously (sc) at 10 mg/kg body weight (groups 3 and 5) and controls (groups 2 & 4) received the same volume of SSO/kg body weight. To supplement or maintain testosterone and SSO in circulation for groups 3 and 5 (T and EDS + T), rats received sc TP on days 0, 2, 4, 6, and 8 while groups 2 and 4 (VEH and EDS) received sc SSO on days 0, 2, 4, 6, and 8 days. Rats, maintained for 10 days, were euthanized under anesthesia and tissue were collected.

Tissue collection

Rats were anesthetized with Isoflurane® and sacrificed by decapitation on the 10th day post EDS. Testicular tissues were excised and washed in phosphate buffered saline (PBS) cold (on ice). Testes were trimmed of excess connective and fatty tissue and weighed. The right testis was used for RNA preparation, protein extraction and preparing isolating cells. The left testis was fixed in modified Davidson's Fluid (mDF) for 24 h at 4 $^{\circ}$C for histological analysis and TUNEL Assay.

Hormone measurements

Trunk blood collected after decapitation of rats was coagulated for 30 min on ice followed by centrifugation at 8300 x *g* for 10 min at 4°C to separate serum from formed elements. This serum was used to measure levels of circulating testosterone. For measurement of intra-testicular testosterone (ITT), after removal of tunica albuginea, approximately 200 mg of testicular tissue was homogenized in 5 ml of 20 mM $NaC_2H_3O_2$ (sodium acetate), pH 5.0, for 1 minute since this pH releases testosterone from androgen binding proteins and allows more accurate measurements of total testosterone from tissue (O'Donnell *et al*. 1994). Tissue homogenates were centrifuged at 8000 x *g* for 10 min at 4 °C and the supernatant were collected for measurement of ITT. Serum testosterone and ITT were both measured with a radioimmunoassay (RIA) kit having a detection limit of 0.05 ng/ml (Testosterone RIA DSL– 4100, from DSL, Webster, TX). Radioimmunoassay is a competitive binding assay where a constant amount of anti-testosterone antibody (known) is coated in the tube provided in the kit. A small amount of radiolabeled testosterone (I^{125}) plus increasing amounts of unlabeled reference standard or sample are added to the tubes. After 1 hour of incubation, the antibody-bound (labeled & unlabeled) testosterone is separated from the free or unbound testosterone. Because a fixed amount of antibody and labeled testosterone is present in each assay tube, the amount of labeled testosterone bound to antibody (the measured radioactivity by Beckman's Gamma 5500) depends on the competitive concentration of unlabeled testosterone in either the standard or samples. That is, the higher the concentration of unlabeled testosterone, the more the

labeled testosterone is displaced from the antibody and thus lower radioactivity is bound to the fixed amount of antibody in the tube. A standard curve is generated by plotting the ratio of the amount of labeled testosterone bound (~25% of added testosterone ($T^{I\text{-}125}$) was considered 100% binding in the absence of unlabeled testosterone. The amount of T^{I125} the absence of any unlabeled testosterone as a function of amount of standard. The standards are plotted for y-axis (log/antilog) percent bound and x-axis (4 cycle log) the range of cold (competitive) testosterone was (0.01 ng to 32 ng). The quantified amount - ng of testosterone/mL serum and ng of testosterone/gm testicular tissue was determined by interpolating values from the standard curve.

RNA extraction

Total RNA was extracted using TRIzol (Invitrogen) in accord to the manufacturer's protocol. For extraction of total RNA, approximately 200 mg of testicular seminiferous tubules from each sample was homogenized in 2.0 mL of TRIzol with an Ultra-Turrax homogenizer for 45 seconds with a small generator probe at 2400-2450 rotations/min in a 15 mL polypropylene tube. The homogenate was incubated for 15 min in a 37°C water bath. Following incubation, 0.2 mL of chloroform/mL of TRIzol (0.4 mL/2 mL that was added to the homogenate and vortexed vigorously. The chloroform containing homogenate was incubated at room temperature for 15 min followed by placing on ice for 10 min then centrifuged at 7500 rpm for 15 min at 4 °C. Centrifugation resulted in separation of the homogenate into two distinct phases, a clearer (aqueous) phase on top of a red layer (organic phase) in the bottom of the tube. The upper aqueous

phase (containing RNA) was transferred to a new tube and an equal volume of 100% isopropanol was added and capped for mixing. The tubes were vortexed and incubated at room temperature for 20-30 min to facilitate the precipitation of total RNA. The total RNA precipitate was collected by centrifugation at 6000 rpm for 10 minutes. The alcohol: water solution was decanted into a small beaker and then discarded in chemical waste. The RNA pellet was washed with 1.0 mL of cold 70% ethanol by vortexing to remove minor traces of phenol. The washed RNA was collected by centrifugation of tubes at 7000 rpm for 10 min at 4 °C to collect the washed total RNA. The (alcohol: water) supernatant fluid was decanted (disposed in organic waste) while leaving a white pellet at the bottom of each tube. The RNA pellets were air dried for 20 min at room temperature to remove any ethanol. Then RNA pellets were dissolved in 500 µl TE buffer [Tris-EDTA (1.0 mM Tris and 0.10 mM EDTA)] and stored at -20°C for further analysis.

Quantification of Total RNA

The total RNAs were diluted 1:100 in TE buffer and subjected to spectrophotometric analysis to assess purity and quantity/yield. The purity was assessed by the A_{260nm}/A_{280nm} ratio with ratios of 1.8-2.1 considered to be pure enough from protein contamination for further analysis. The extinction (absorption) coefficient of 25 A_{260nm}/mg RNA was used to determine total RNA concentration in each sample. By multiplying the A_{260nm} x 100 the concentration of the original isolate is corrected to 0.25 ug/ul by dilution with TE buffer. For gel electrophoresis, 3.0 µg of RNA from each extract was subjected to gel electrophoresis in 1.5% agarose (molecular biology grade)

with 40 mM Tris-Acetate, 1.0 mM EDTA (pH 8.1) buffer and stained for an hour with 0.5 µg/mL of ethidium bromide. The quality and integrity (intactness) of RNA was analyzed by measuring the fluorescence of rRNA bands by calculating the ratio of 28S/18S bands with the help of Alpha Innotech's FluorChem HD2 gel imaging software. While fluorescent ratios of 28S to 18S are expected to be ~2, we have found that ratio's >1.1-1.5 work well and was considered to be integrity sufficient for further analysis using RT-PCR.

Reverse transcription (RT)

Total RNA (2.0 µg) from each testicular RNA was used to make complementary DNA (cDNA) using Invitrogen SuperScript III® First-Strand Synthesis kit for reverse transcription in a thermal cycler. Two micrograms of total RNA was primed using 50 µM oligo-dT_{20} (to target the poly-A tail of mRNA) in a total volume of a 21 µL reaction. After cDNA synthesis, the final RT reaction was diluted 25-fold using 25 µg/mL of acetylated-BSA (RNAse and DNAse free-Invitrogen) as a carrier for the diluted cDNA for real-time quantifiable polymerase chain reaction (rt-qPCR).

Primer design and function

Since DNA polymerase requires primers with an open 3'-OH to initiate DNA synthesis - strand extension, (using Primer 3) selected genes were targeted by designing gene-specific primers of 17 to 23 nucleotides for both forward (+) and reverse (–) strand of the DNA encoding specific genes. The primers were designed to produce short dsDNA 250 to 300 base pairs (bp) in length to keep cycle times short. Primer sets are localized

more to the 3' ends of the specific genes (mRNAs) to be studied, since the cDNAs synthesized by RT are oligo-dT primed at the 3’ end and may copy more of the mRNAs with cDNA extension in that region of the mRNAs for longer messenger RNAs. Each primer had a ½ T_m of 62-66 °C and 40-60% GC content. The product lengths of the amplified cDNAs were approximately 250 to 300 bp to facilitate comparison of cycle numbers for comparing relative expression abundance. The primer sets for the targeted region of the genes were picked from two different exons to ensure that the amplified cDNAs were only from the original cDNAs and not from any genomic DNA that might be present as a contaminant from the RNA extraction. If the genomic DNA is amplified, the product lengths will be longer (product comprised of lengths of exons and introns) than the fragments obtained from the amplification of cDNAs. All primers were designed and validated using NCBI-PUBMED software. Primer sequences of the genes for this study are listed in Table 1.

Table 1

Primer sequences of genes of interest

Gene	GenBank® Accession Number	Primer Sequences	Product Length
Hsd3β2	NM_0107265.4	**Fwd:** TGGAGCAGCACAGGGAGACA **Rev:** GACATGCTTTGGATGGGGAGAGA	300 bp
Inls3	NM_053680	**Fwd:** TGCAGTGGCTGGAGCAACGACATC **Rev:** TTCATTGGCACAGCTGTNAGGTGGG	265 bp
Fas-R	NM_139194.2	**Fwd:** TGAGGGATGACCGTCTTGGCTGTCC **Rev:** CATTCCTGGAGCCTCTGGGTGAAGC	241 bp
Fas-L	NM_012908.1	**Fwd:** ACAGGAGGCCGTCTTTTAGACACATGG **Rev:** CACACACAGACATCATTGCACTGGAGG	242 bp
FADD	NM_152937.2	**Fwd:** CGAAGCCTGAGTGATCGGGTAAGGG **Rev:** TGAGAGGAGCCTCGGGCTTGTCAG	245 bp
Tnf-α	NM_012675.3	**Fwd:** CGTCGTAGCAAACCACCAAGCGGA **Rev:** GCTCTTGATGGCAGAGAGGAGGCTGAC	250 bp
Bad	NM_022698.1	**Fwd:** TCCGAAGAATGAGCGATGAATTTGAGG **Rev:** GAAAGACTTCCGCCCACACCCAATATG	234 bp
Bax	NM_017059.2	**Fwd:** AGGATCGAGCAGAGAGGATGGCTGG **Rev:** AACATGTCAGCTGCCCACCCGGAAG	205 bp
Bak-1	NM_053812.1	**Fwd:** CTTTGCGGCTGGACTCTCAGGGATTC **Rev:** TAGCGTGAGCACCTCCCACTGAATGC	240 bp
Bik	NM_053704.1	**Fwd:** TAGCCTGCATCGGCGATGAGATGG **Rev:** ACCAGCAGCACCATGGGAAACAGC	247 bp
Bok	NM_017312.2	**Fwd:** AACCCCATTCCTTTGTGGACTCTGGC **Rev:** CAAAGACTGAGGCCAGCTCATGTCCTG	239 bp
Caspase - 3	NM_012922.2	**Fwd:** TGAGAGCGTAAGGAAAGGAGAGGTGGC **Rev:** TTGAGATGCTCAGCACTCTGGGAAAGC	217 bp
Caspase - 8	NM_022277.1	**Fwd:** GATGAGGCAGACTTTCTGCTGGGGATG **Rev:** CTTCCGTAGTGTGAAGATGGGCTGTGG	233 bp

Caspase - 9	NM_031632.1	**Fwd:** ATCACTGCTTCCCAGACCCACAGTCC **Rev:** TCAGGCCGAGACCTTGGAACACAGAG	243 bp
Bcl – 2	NM_016993.1	**Fwd:** CTTCCAGCCTGAGAGCAACCGAACG **Rev:** TGGACATCTCTGCAAAGTCGCGACG	191 bp
Bcl – W	AY_185098.1	**Fwd:** CTGCTGAGCCTCCAGACAGGTGAAAGG **Rev:** TGGCCAAGAGCTGATCCCAAGGAAC	227 bp
Mcl – 1	NM_021846.2	**Fwd:** TCCTTGTCTGGAGATGGAAGAGTGGCC **Rev:** GCTCAGAGCACATCCTCCTTCACCCTC	210 bp
Bcl – XL	NM_001033672.1	**Fwd:** CCATGGTGACCATGACTGAGGGACC **Rev:** TTCCTGCCCTTCCTGCCTGAGGTAG	227 bp
Bcl2L10	NM_053733.1	**Fwd:** CCTGTTTCTTTGCAACGGCCA **Rev:** TCTAGTGAGTGGGTGCAGCG	275 bp
Itgα6	NM_053725.1	**Fwd:** TCAGGGATAGCGTGGTGGATCA **Rev:** TTTCCGAATCCGCACCGCAT	283 bp
CD 9	NM_053018.1	**Fwd:** TGGCATAGCTGGTGGTGTGG **Rev:** AATGGCCCGACTGTCCTCAGA	282 bp
Ldh C4	NM_012978.1	**Fwd:** CCTTCTGCTGCTGAGCCGAT **Rev:** GGCCGTCCTCTGAAGCAGGTA	291 bp
Tnp 1	NM_017056.2	**Fwd:** ACCGAGCTCCTCACAAGGGC **Rev:** TGTTGGGGAGAAACAGCCAACA	300 bp
Tnp 2	NM_017057.2	**Fwd:** CCACCCGAGCTCCAGTTCCA **Rev:** CCCAACATTCCCCTAGTGATGGC	289 bp
Paqr 7	NM_001034081.1	**Fwd:** CGAGGAGGATCCCGCCCTTC **Rev:** GTTGTGGGGCCAACGGG	250 bp
Paqr 8	NM_001014099.1	**Fwd:** TGGAGTGGACTGCACCTCTGT **Rev:** TGCGTGGATGTACGGCTCCC	279 bp
Paqr 5	NM_001014092.1	**Fwd:** CTTCGCCTACCCCTACGCCT **Rev:** TGTGTGGCCAGGATGACGCA	234 bp
Paqr 3	NM_001012033.1	**Fwd:** TCTTCTGCTCGGTGTCGGGA **Rev:** CACTGCAAGGACGTGCCAGAT	234 bp
Paqr 9	NM_001271152.1	**Fwd:** CAAAGACGCAAACCCGGGGC **Rev:** TGCCCTAATGCCCGGCTCAC	222 bp
Pgrm C1	NM_021766.1	**Fwd:** TGGCCATCAACGGCAAGGTGT **Rev:** CAATCGGCTCCTCCGCTCCTT	270 bp
Gapdh	NM_017008	**Fwd:** TGAACGGGAAGCTCACTGGCATGG **Rev:** CAATGCCAGCCCCAGCATCAAAG	234 bp

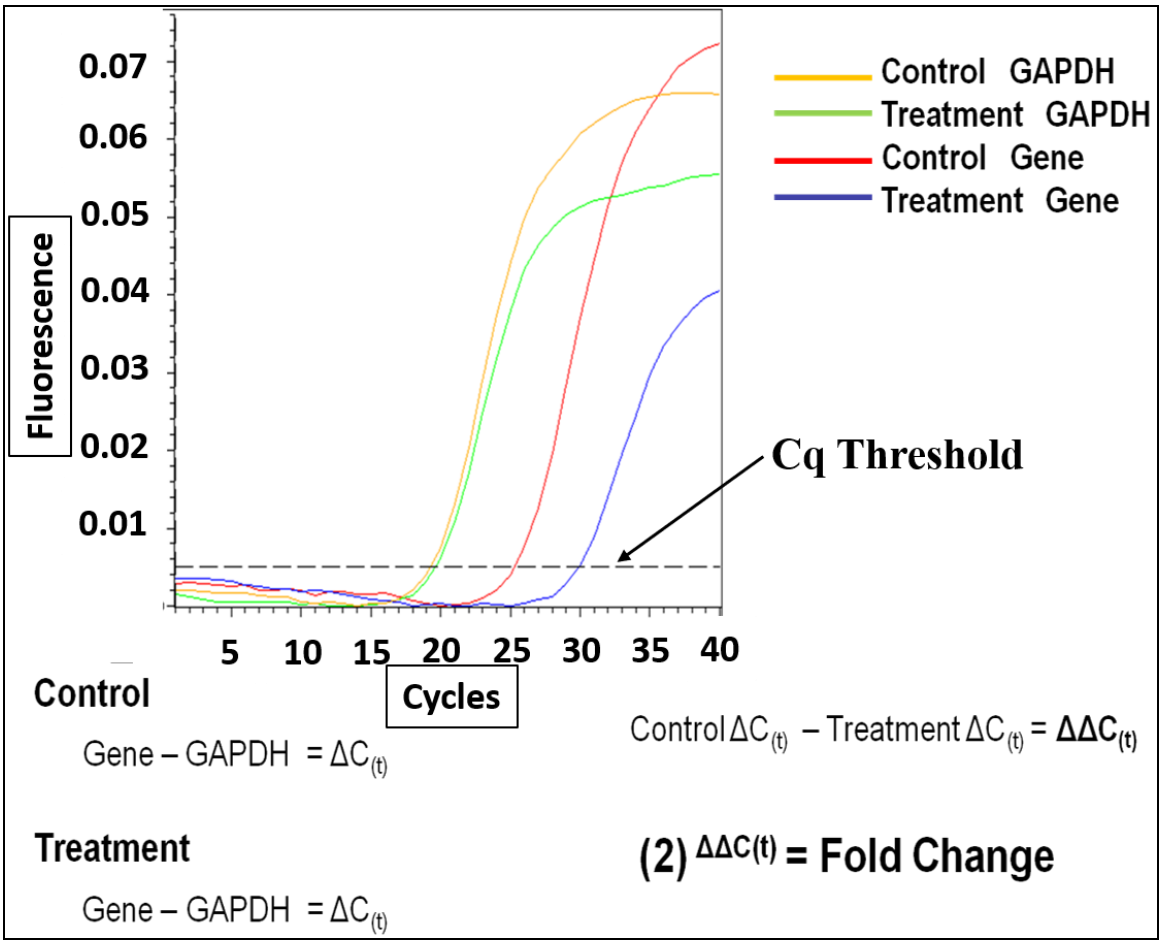
Quantifiable real-time polymerase chain reaction (qrt PCR)

Quantifiable real-time PCR (qrt PCR) was conducted using SYBR Green Master Mix kit in the Biorad CFX96 for quantification of mRNA abundance. A 25.0 µl reaction mixture was made by adding 13.0 µl of 2X SYBR Green Master Mix, 1.5 µl of 10 µM forward primer, 1.5 µl of 10 µM reverse primer, 6.0 µl of the diluted cDNA (1:25) sample from the RT reaction and 3.0 µl of deionized H_2O. Thermal cycling was set at 95°C for 10 min for the activation of the Taq polymerase. Then 40 cycles of template denaturation at 94°C for 30 sec, primer annealing at 60°C for 25 sec, extension for 30 sec at 72°C and fluorescence reading at 78°C to determine cDNA production were performed. After 40 cycles, the temperature was ramped slowly to obtain the melting curve of the amplified dsDNA product to establish the quality of the dsDNA product. That is, the accuracy of the dsDNA synthesis. The qrt PCR products were further assessed by agarose gel (1.5%) electrophoresis. For each gene, one band is expected in each lane at their respective product sizes. Reactions with no template were run concurrently as negative controls and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the housekeeping gene. The difference in quantity of the mRNAs expressed were performed by the $2^{(-\Delta\Delta Cq)}$ method (Kubista et al. 2006).

Gapdh was selected for the tissue reference gene after it was found to change little in all of the treatments that were used in the study. *Gapdh* exhibited little variability in the standardized approach of RNA purification and reverse transcription and with the standardized dilution of the RT reaction, it normally appeared around 19 cycles of PCR.

Figure 34

Calculation of relative abundance of mRNA by reverse transcription and quantifiable real-time PCR (RT-qrtPCR).



Note. A specific fluorescence level, for quantification is chosen, that is set above the passive background fluorescence of the dye, carboxyrhodamine (ROX) but at lower cycle numbers of the samples at exponential linear amplification of dsDNA as measured by fluorescence of Sybr Green when it binds directly in the small groove of the dsDNA. The cycle intercepts of genes being amplified are used for cycle quantification $C_{(q)}$

selected for the genes being compared. The $C_{(q)}$ value of the target genes is subtracted from the $C_{(q)}$ value of the sample internal reference gene (housekeeping gene (*Gapdh*)) that gives the difference between the sample $C_{(q)}$s ($\Delta C_{(q)}$) for the control sample. Similarly, the $C_{(q)}$ value for the target genes in the treatment group is subtracted from the $C_{(q)}$ value of the housekeeping gene (*Gapdh*) from the treatment group and gives the $\Delta C_{(q)}$ for the experimental sample. The control group $\Delta C_{(q)}$ is then subtracted from the treatment $\Delta C_{(q)}$, giving the differences between the 2 samples or $\Delta\Delta C_{(q)}$s for the gene being analyzed. Since, cDNA is single-stranded, and with PCR they are being amplified to double-stranded DNA, the difference in the number of copies of mRNA is determined by $(2)^{-\Delta\Delta C(q)}$ computation to convert the differences to a total numerical value.

Tissue Protein Extraction and Quantification

Testicular tissue (~200 mg) was extracted using 2.0 mL of lysis buffer. We estimated the protein concentration using Thermo Scientific™ Pierce™ BCA Protein Assay kit in accordance with the manufacturer's protocol. Thereafter, we evaluated the concentration of testosterone in the treatment groups using Cayman Testosterone ELISA kit following the manufacturer's instruction. The measured concentration was normalized using the protein concentration in each sample.

Western Blotting

Protein samples, together with marker proteins (Precision Plus Protein unstained standards from Bio-Rad), were separated on TGX Stain-Free gels (Bio-Rad) for 90 mins at 120 V. The gel was then UV activated for the Stain-Free protein detection procedure

and imaged with the ChemDoc (BioRad). The activated gel was transferred to an Immun-Blot low-fluorescence polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in 30 min using the Trans-Blot Turbo Transfer System (Bio-Rad) with Trans-Blot Turbo Mini Transfer Packs.

The Immuno-Blot PVDF membrane was blocked for 2 hours at room temperature with gentle agitation using BSA blocking buffer for fluorescent Western blotting (Rockland, USA). Primary monoclonal antibodies were mixed in phosphate buffered saline (PBS, pH 7.4) with 5% bovine serum albumin and incubated for 3 hours at room temperature. After incubation with primary antibodies, the blotting membrane was washed for 10 min with PBS. Secondary antibodies were mixed in PBS with 5% bovine serum albumin and incubated for 1 hour at room temperature. After incubation with secondary antibodies, the blotting membrane was washed for 10 min with PBS and dried overnight between two sheets of filter paper. After antibody incubation steps, band intensity was detected via fluorescence, imaged via ChemiDoc MP (Biorad) and band intensity quantified using Image J software.

Fixative for Histology

For preparation of modified Davidson's fixative (mDF), 30 ml of 37% formalin (11% formaldehyde), 15 ml of absolute ethanol (15%) and 5 ml glacial acetic acid (0.9 M) were added to 50 ml of deionized water and mixed (Garrity 2003; Lanning 2002; Latendresse 2002; Stahelin 1998; Tornusciolo 1995). Modified Davidson's fixative (mDF) had a pH of 3.40. Since methylene hydrate, the reactive form of formaldehyde in

water is slow to form at lower pH, (Kiernan 1990) mDF was prepared several days in advance of usage.

Slide Preparation

Sectioned testicular tissues are difficult to maintain on glass slides for extended procedures and proteinase K digestions; therefore, slides were routinely cleaned and coated (subbed; Shuttlesworth & Mills 1995). Glass slides (Fisher) were cleaned by soaking in a solution of 0.2 N HCl and 95% ethanol for 20 minutes, followed by rinsing with tap water and distilled water, sequentially. Slides were air dried after cleaning. To prepare the chromate-collagen mixture for subbing, 0.5 gm of bovine gelatin (EM Science, Gibbstown, NJ, USA) was dissolved in 80 ml of warmed deionized water. To that solution, 0.50 ml of Hipure liquid gelatin (cod fish skin, Norland Products, North Brunswick, NJ, USA) was added. After cooling, 20 ml of deionized water containing 0.10 gm of dissolved chrome alum or chromium potassium disulfate dodecahydrate [$(CrK(SO_4)_2 \cdot 12H_2O$] (EM Science) was added to the gelatin mixture (Shuttlesworth & Mills 1995). Slides were subbed with the chromate-gelatin only on the frosted side with a cotton swab. Subbed slides were air dried overnight at room temperature and stored in slide boxes at refrigerated temperatures until use. Subbed slides in boxes were set at room temperature before use to prevent moisture accumulation between the sections and subbed surface of the slides.

Tissue Processing

After dissection, intact testes were immersed into mDF with tissue to fixative ratio of 1:10 (weight: volume) and were refrigerated. Prior to fixation, testes were pierced superficially at 5-6 locations on both ends with a 23-gauge needle to facilitate fixative permeation (Latendresse 2002). After immersion for 2 to 3 hr, testes were trimmed into 3 to 5 mm pieces and fixation was continued for 24 hr in the refrigerator (4°C). After fixation, testes were dehydrated in the refrigerator with ascending concentrations of 70%, 90%, and 100% ethanol for 2 hr each. At room temperature, testes were cleared by immersion in a 50:50 mix of xylene-ethanol for 2 hr followed by two changes of xylene for 2 hr each. For paraffin embedding, testes were first transferred to a mix of 50:50 molten paraffin wax-xylene and incubated at 60°C for 1 hr followed by three changes of pure molten paraffin wax for 1 hr each at 60°C. Each piece of testis was transferred to warmed stainless steel tissue molds for paraffin wax embedding. From each tissue block, 7 µm thick sections were cut with a rotator microtome and the ribbons floated in a 37°C water bath to permit section spreading. The sections were collected onto the subbed slides and allowed to dry and attach to the subbed slides by warming the slides at 40°C for 15-20 min on a slide warmer. The slides were refrigerated in a slide box until use.

Hematoxylin and Eosin (H&E) Staining

For H&E staining, the sections were first dewaxed with xylene followed by rehydration in descending concentration of ethanol (100%, 90%, and 70%) for 15 min each at room temperature. Rehydrated sections were stained with Modified Mayer's

hematoxylin (American MasterTech; diluted 1:1 with distilled water) for 3 min at room temperature. Sections were counterstained with eosin (0.10% Eosin Y; 0.50% glacial acetic acid v/v dissolved in 1.0 L of 70% ethanol) for 3 min at room temperature. After dehydration of stained sections in increasing concentrations of ethanol (70%, 90%, and 100%) for 3 min each followed by 5 min in xylene, sections were covered with Permount mounting medium and mounted with coverslips.

Image Capture and Cell Counting

All images were captured using the Nikon's eclipse 90i digital microscope at a voltage of 9.2 and exposure time of 4 ms. Areas of seminiferous tubules were measured with Nikon's NIS-Elements Basic Research version 3.1 software's annotation and measurement tool. Adobe Photoshop 7.0 was used to adjust the brightness and contrast of the images for visual presentation.

Immunohistochemistry (IHC)

For detection for germ cell nucleus, testes sections were dewaxed in five changes of 100% xylene for 5 minutes each, rehydrated in 100%, 90%, and 70% ethanol and then boiled in 1.0 L of 1.0 mM EDTA (pH 7.5) for 15 min in 800-Watt microwave for antigen retrieval (McCabe et al., 2010). Sections were mounted in Fluoroshield with DAPI (Sigma) and nuclei was visualized using Nikon's eclipse 90i digital microscope.

For detection of androgen receptors, testes sections were dewaxed in 5 changes of 100% xylene for 5 minutes each, rehydrated in 100%, 90%, and 70% ethanol and then boiled in 1.0 L of 1.0 mM EDTA (pH 7.5) for 15 min in an 800-Watt microwave for

antigen retrieval. Non-specific antigenic sites were blocked by pre-incubating sections in 10% normal donkey serum (Jackson Immunoresearch) in PBS for 1 hr in a 37°C humidified chamber. Rabbit anti-Androgen Receptor (1:50 from stock concentration 200 µg/ml; Santa Cruz Biotechnology) in 10% normal donkey serum in PBS as primary antibody was applied to the sections and incubated in a humidified chamber at room temperature (23°C) for 18 hr. Isotype matched non-specific mouse immunoglobulin 1 (IgG1) or 10% normal donkey serum respectively was used instead of primary antibody for negative controls. Primary antibodies were detected with 1:500 dilution of Donkey anti-rabbit DyLight 649 (Jackson Immunoresearch) in 10% normal donkey serum in PBS. Coverslips were mounted with Vectashield mounting medium containing 4' 6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and immunofluorescence images were captured with Nikon's Eclipse Ti confocal microscope.

Germ Cell Isolation

Ten day treated rats were dissected and the testis was removed and collected in ice cold PBS. The tunica albugenia was removed and tissue was transferred into small culture plates with Digestion medium (DMEM/F-12 with Collagenase, Hyaluronidase, Dnase I and PEN-STREP). Each tissue was minced with the help of scissors (first) and teased with needles until all seminiferous tubule pieces were less than a millimeter. After a brief incubation for 25 mins at 37ºC with slow continuous shaking, the media are carefully triturated to release the germ cells in a homogenous solution. The cell suspensions were filtered using 40 µm filter and centrifuged at 400 g for 10 mins (4ºC).

Discarding the supernatant fluid, the pellet was re-suspended in cold PBS and counted to 1 X 10^6 cells/ml.

Flow Cytometric Analysis

1.0 x 10^6 germ cells were stained with 10 µM final concentration of Vybrant DyeCycle Green (Invitrogen) for distinguishing cell populations according to DNA content. Non-permeant dye Propidium Iodide (PI) at a final concentration of 10 µg/ml and 37ºC incubation for 30 minutes in darkness was used to distinguish live germ cells (negatively stained for PI) from dead germ cells (positively stained for PI). At least 10,000 events were analyzed per sample in Cytoflex Flow Cytometer (Beckman Coulter) and data were analyzed using CytExpert 2.3.0.84 software (Beckman Coulter).

Competitive Binding Assay

For competitive binding of mPRα and testosterone, 1.0 x 10^6 germ cells were suspended in PBS with freshly added SIGMAFAST Protease Inhibitor Cocktail tablets, EDTA free (Sigma). Cells were cold centrifuged at 1500 rpm. Pelleted germ cells were homogenized in cocktail of PBS and protease inhibitor. 100 µl of germ cell suspension was pipetted on coverslips in 12 well plates and incubated at 37ºC for 30 minutes. Post incubation, the cells were centrifuged at 500 g for 5 minutes and unbound cells on coverslips were removed via washing and removal of supernatant. To induce competitive binding, germ cells were incubated at room temperature at a final concentration of 10^{-7} M testosterone 3-(O-carboxymethyl) oxime: Bovine serum albumin – fluorescein isothiocyanate conjugate (testosterone BSA-FITC;Sigma) and 10^{-7} M progesterone 3-(O-

carboxymethyl) oxime : bovine serum albumin – fluorescein isothiocyanate conjugate (progesterone BSA-FITC; Sigma) for 30 minutes in presence and absence of 10^{-6} M cold testosterone 3-(O-carboxymethyl) oxime (Sigma). As controls, 1:1000-fold dilution of BSA-FITC (Sigma) was used to determine nonspecific binding. Post incubation, cells were then washed twice and fixed with 2% paraformaldehyde in PBS for 30 mins. Coverslips were mounted onto slides using Fluoroshield with DAPI (Sigma). Slides were visualized using a upright BX51 epifluorescence microscope and image analysis done with ImageJ software.

Statistical Analysis

Data for mRNA levels for genes from all treatment groups were normalized to the untreated (NT) group and were compared by 3-way analysis of variance (ANOVA) with GraphPad version 8 software. Comparisons were between EDS treatment (EDS), testosterone supplementation (T) and testosterone replacement (EDS + T), and effect of 10 days. Comparison between untreated (NT) and vehicle (VEH) groups were performed by Student's t-test. To compare concentrations of serum testosterone, 2-way ANOVA, and to compare ITT concentrations, 1-way ANOVA was performed. Post-hoc comparisons were performed with Newman-Keuls test and p-value ≤ 0.05 was considered significant (Zar 1999). All data are reported as mean $\pm$ standard error of mean (SEM), and graphs were generated by GraphPad version 8 software.

CHAPTER III

RESULTS

This study investigated the role of testosterone on germ cell viability and germ cell apoptosis following testosterone withdrawal. The principal questions addressed were:

1. Is spermatogenesis a testosterone dependent process?
2. Is testosterone dependent germ cell apoptosis targeted to particular germ cell population(s)?
3. How does testosterone impact the alternate pathway, if any, of testosterone action(s) in germ cells?

To answer the above-mentioned questions, gross morphological and histological evaluations were performed to study the effects of testosterone depletion by ablating Leydig cells that are the primary source of testosterone in the male. Gene expression analysis, western blot and immunohistochemistry for protein localization have been performed to decipher the effects of testosterone depletion in testes. We have developed supporting evidence for the presence of a non-canonical pathway for testosterone action in germ cell populations that comprise 80% of testicular cell populations using flow cytometry and competitive binding assays.

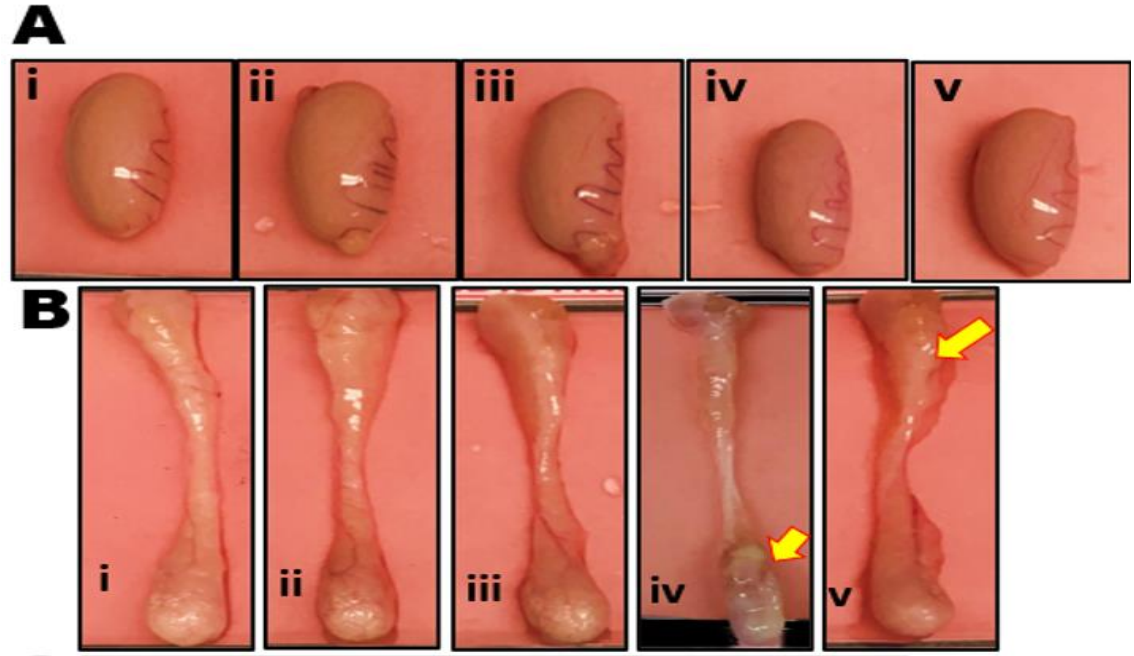
Is Spermatogenesis a Testosterone Dependent Process?

Testosterone Withdrawal Significantly Effects Testicular and Epididymal Morphology and Weight

Gross morphology of testes did not appear different between the five control and treatment groups after 10 days; however, EDS treated testes appeared slightly smaller than testes of other treatment groups (see Figure 35 A). However, the epididymis of EDS treated/testosterone depleted rats had distinct sperm granulomas (white lesions, marked by arrowheads) as observed in the cauda epididymis at 10-days post EDS treated rats and caput epididymis of EDS treated-testosterone replaced rats. (see Figure 35 B; iv & v, respectively). The body weights were tabulated as there was no change in mean body weight between the treatment groups and control groups (see Figure 35 C-iv). There was a significant reduction in total testis weight among EDS-treated rats compared to the controls. The average loss of testis weight was 25% after 10 days of treatment. Leydig cells constitute only 2.7% of the total testicular volume in the adult rat testis (Mori & Christensen 1980), so loss of 25% weight can be due to the loss of germ cells as well. Testes weights remained at the control levels in rats receiving testosterone replacement after EDS treatment (EDS+T; see Figure 35 D). Epididymal weights of EDS-treated rats were reduced 33% compared to controls (see Figure 35 E).

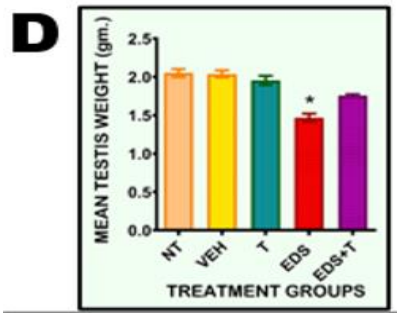
Figure 35

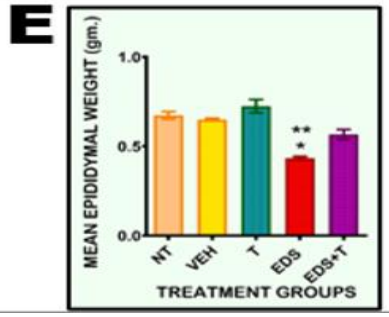
Testosterone withdrawal significantly effects testicular and epididymal morphology and weight.



C

NT	VEH	T	EDS	EDS+T
540	520	510	470	490
483	583	561	533	517
550	540	500	522	513
549	554	542	480	514
500	559	552	470	500
494	441	453	432	433





Note. (A) Representative images of gross morphology of rat testes after 10 days of treatment. (i) Not Treated (NT); (ii) VEH; (iii) Testosterone supplemented; (iv) EDS-treated; (v) Testosterone replaced after EDS-treated. (B) Gross morphological appearance of rat epididymis after 10 days of treatment. (i) Not Treated (NT); (ii) VEH; (iii) Testosterone supplemented; (iv) EDS-treated; (v) Testosterone replaced after EDS-treated. (C) Table of body weights (gram) among the groups of controls and treatments 10 days post-EDS treated rats (n = 6). (D) Mean testis weights of the 5 - control and treatment groups at 10 days post-EDS treated rats (n = 6). Asterisk (*) indicates significant difference from all other treatment groups. (E) Average epididymis weights of 10 days post EDS treated rats. Error bars represent $\pm$ SEM with n = 6. Asterisk (*) indicates a significant difference from Not Treated rat group, vehicle treated rat group and testosterone supplemented group. Asterisks (**) indicates a significant difference from testosterone supplemented group.

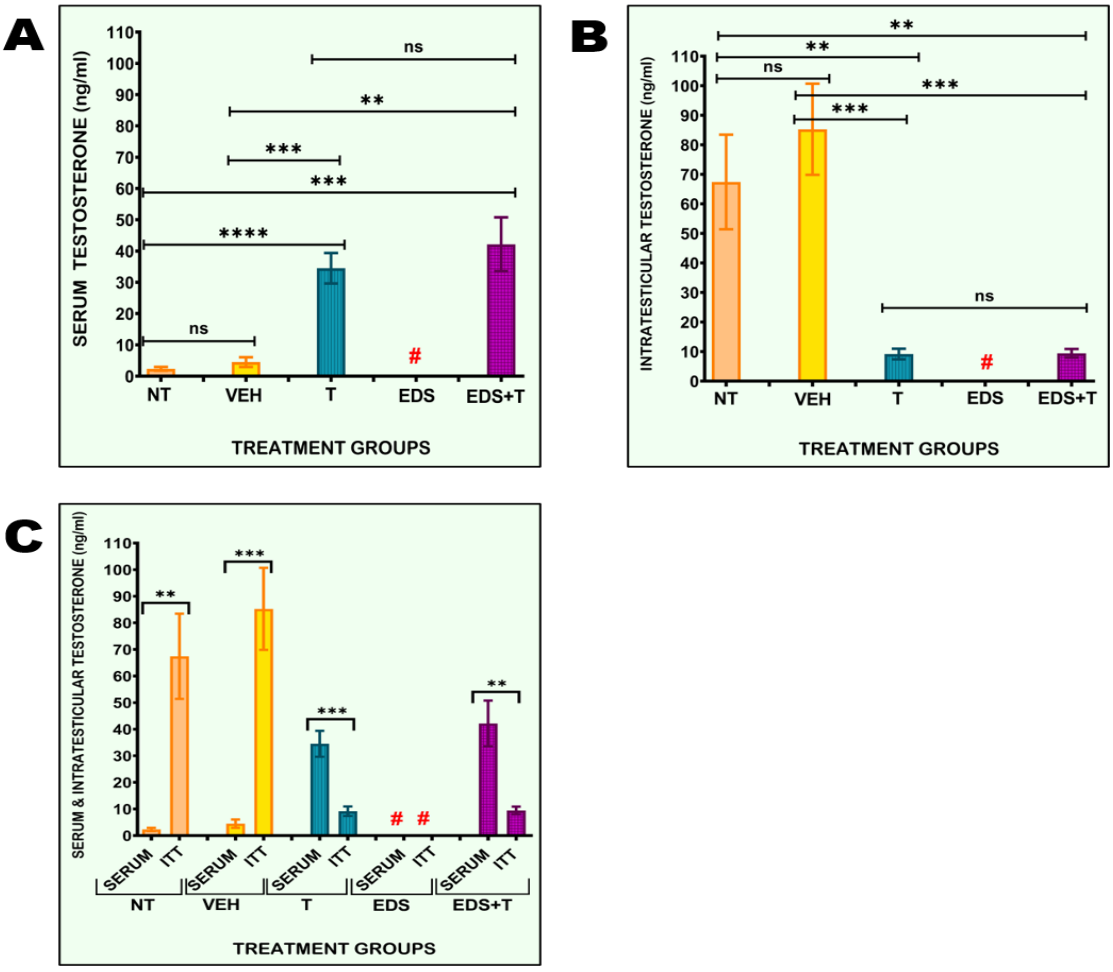
Testosterone Concentrations in Serum and Testis is Undetectable Upon EDS Treatment

EDS induced elimination of testosterone producing Leydig cells indicates an ablation of testosterone. Testosterone was undetectable (ND < 0.05 ng/g) in serum or testicular tissue in 10-days post-EDS treated rats, which supports our hypothesis. Normal serum testosterone levels were observed in our experimental controls; Not Treated (NT) (2 ng/ml) and vehicle treated (VEH; 4.5 ng/ml) rats. Exogenous administration of testosterone propionate increased the serum testosterone levels by 7 to 10-fold above normal in the T and EDS+T treatment groups (see Figure 36).

The concentration of intratesticular testosterone (ITT) has been reported to be between 60–100 ng/gm of the testis (Sharpe 1994). The average levels of ITT in this study were 68 and 85 ng/g of testis in NT- and VEH-treated rats, respectively, and is consistent with previous literature (Sharpe 1994). On the contrary, even with a 7 to 10-fold elevation in serum testosterone levels, ITT in T and EDS+T groups was 6 to 10- fold lower, compared to the NT and VEH treatment groups, respectively. Although complete restoration of ITT was not observed, the average concentration of ITT in the EDS+T group was comparable to the testosterone-supplemented (T) group (see Figure 36).

Figure 36

EDS treatment induced undetectable levels of testosterone concentration both in serum and testes.



Note. (A) Serum testosterone levels, (B) Intra-testicular testosterone levels, (C) Comparison of serum and intra-testicular testosterone. Error bars represent SEM with *n* = 6. In EDS-treated rats, serum testosterone and ITT were below the detection level (0.05 ng/ml) of the assay (ND). Hashtags (#) indicate undetectable level of serum testosterone concentration and ITT concentration in EDS treated rats.

EDS Treatment Significantly Reduced Gene Expression of Leydig cell Markers: Insulin-like Peptide 3 (Insl3) and 3-β Hydroxy Steroid Dehydrogenase (HSD3β2)

Undetectable testosterone in both serum and testes was assayed by ELISA suggested elimination of Leydig cells, and complete elimination was confirmed with RTqPCR. Two Leydig cell-specific mRNAs (Hsd3β2 and Insl3) were quantified by qPCR to determine the degree of Leydig cell loss.

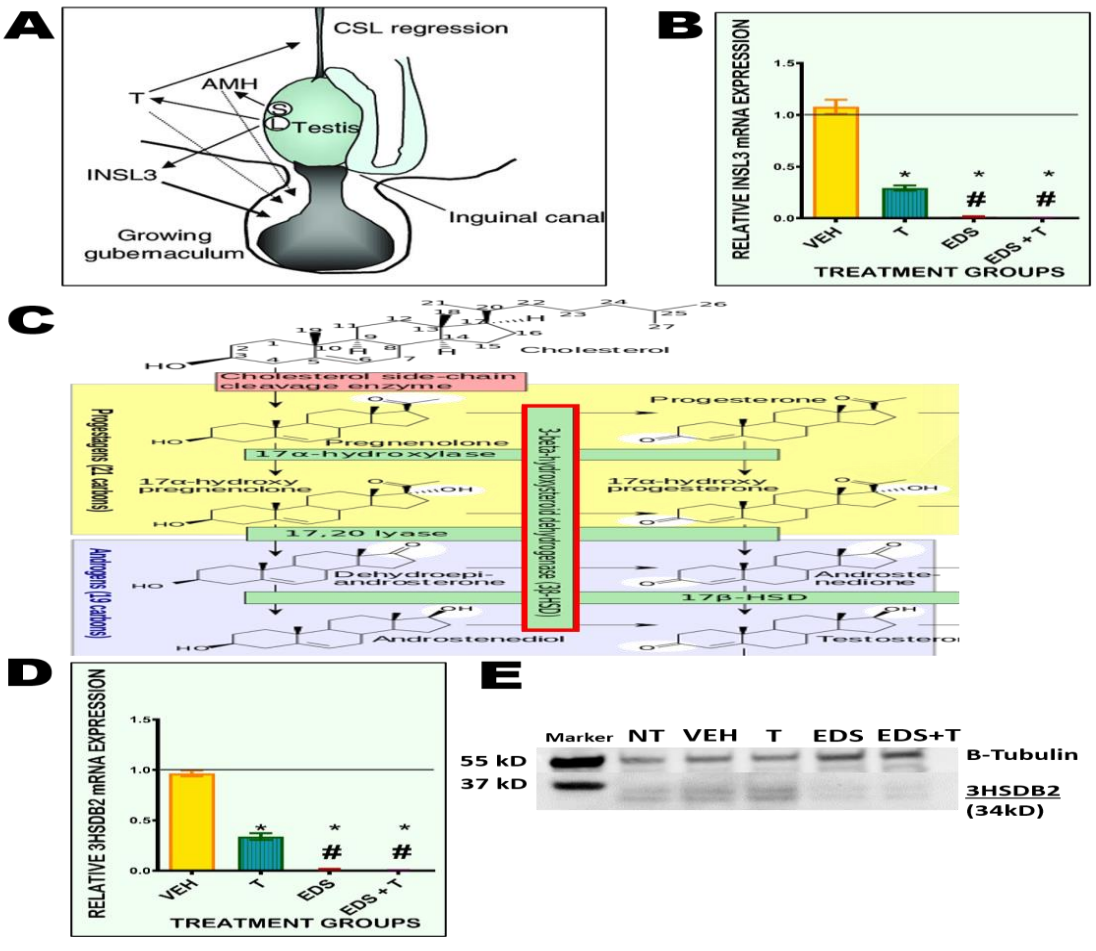
EDS-induced elimination of Leydig cells was confirmed by quantifying the mRNA levels of insulin-like peptide 3 (Insl3), a Leydig cells specific marker in the testis (Koeva et al. 2008; Mendis-Handagama et al. 2007). Testicular descent is regulated by Insl3, which is a member of the insulin-like family (see Figure 37 A; Foresta e al. 2008). Insl3 reduced to < 2% of control (NT and VEH) in EDS-treated rats (see Figure 37 B). Testosterone replacement in EDS+T rats did not improve the level of Insl3 like results from EDS-treated rats. Testosterone supplementation caused 65% reduction in Insl3 gene expression compared to control groups (NT and VEH) may be due to the negative feedback loop from the pituitary gonadal axis (see Figure 37 B).

3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD) is an enzyme that catalyzes the biosynthesis of steroid progesterone from pregnenolone, 17α-hydroxyprogesterone from 17α-hydroxypregnenolone, and androstenedione from dehydroepiandrosterone (DHEA) in the adrenal gland. It is the only enzyme in the adrenal pathway of corticosteroid synthesis that is not a member of the cytochrome P450 family (see Figure 37 C; Cravioto 1986).

The relative amount of mRNA for 3HSD3β2 was 95% reduced 10-day EDS-treated rats. The level of *Hsd3β2* mRNA was further reduced by 7% after testosterone replacement (EDS + T). Testosterone supplementation (T) also resulted in significantly lower 3HSDβ2 mRNA levels in testis compared to vehicle (VEH) in 10-day rats (see Figure 37 D). Results were confirmed by western blots.

Figure 37

EDS treatment significantly reduced gene expression of Leydig cell markers: Insulin-like peptide 3 (Insl3) and 3-β hydroxy steroid dehydrogenase (Hsd3β2).



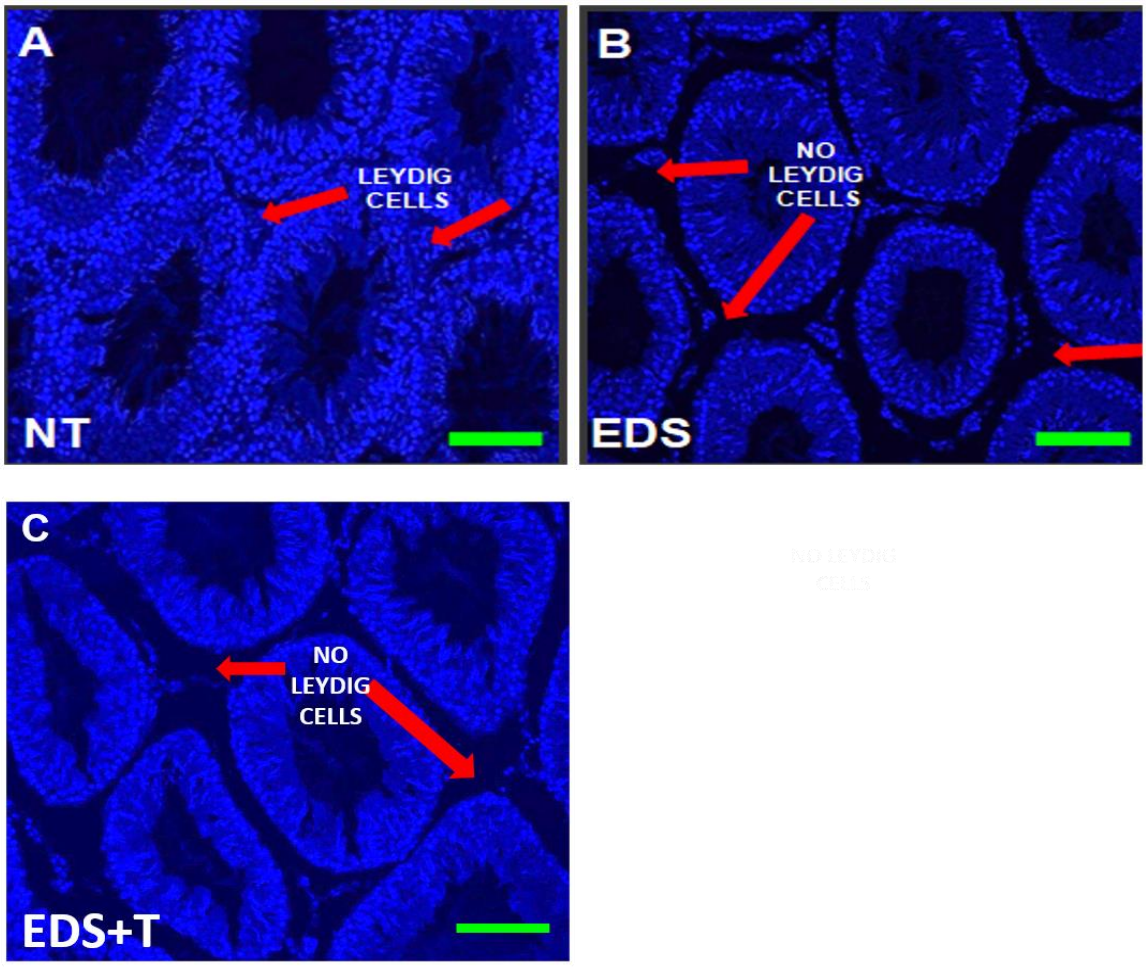
Note. (A) Model of testicular descent in humans, showing the INSL3-dependent transabdominal phase and the androgen-dependent inguinoscrotal phase. The major structures and the roles of hormones are shown. Testicular differentiation from the ambisexual gonad in the presence of the Y chromosome led to the production of anti-Mullerian hormone (AMH) from the developing Sertoli cells (S) and production of testosterone (T) and INSL3 from the Leydig cells (L). (B) Gene expression of *Insl3* using *Gapdh* as the reference gene. Error bars represent SEM with $n = 6$. The horizontal line represents the value of *Insl3* for no treatment (NT) rats normalized to 1.0. Single asterisk (*) indicates significant difference from VEH. (C) Human steroidogenesis, showing reactions of 3β-HSD in middle red bordered green box. (D) Gene expression of 3HSDβ2 using Gapdh as the reference gene. Error bars represent SEM with $n = 6$. The horizontal line represents the value of 3HSDβ2 for Not Treated (NT) rats normalized to 1.0. Single asterix (*) indicates significant difference from VEH. (E) Protein expression of 3HSDβ2 in western blot analysis using β- tubulin as the reference gene.

Absence of Leydig cells in Histological Cross Sections of Testis Following EDS Treatment

Leydig cells reside in the interstitium of rat testes (Kerr 2006). The detection of interstitial Leydig cells was determined by staining Leydig cell nuclei with Dapi. Nuclear staining with Dapi confirmed absence of Leydig cells in the interstitium between seminiferous tubules of EDS and EDS + T testicular cross-sections (see Figure 38). Our results are consistent with the hormonal measurement and mRNA quantification. As controls, normal Leydig cells were observed in cross-sections of normal no treatment (NT) rat testis.

Figure 38

Nuclear staining with DAPI confirmed absence of Leydig cells in the interstitial tissue between seminiferous tubules of EDS and EDS + T testicular cross-sections of 10 day rat testis when compared to no treatment controls (NT).



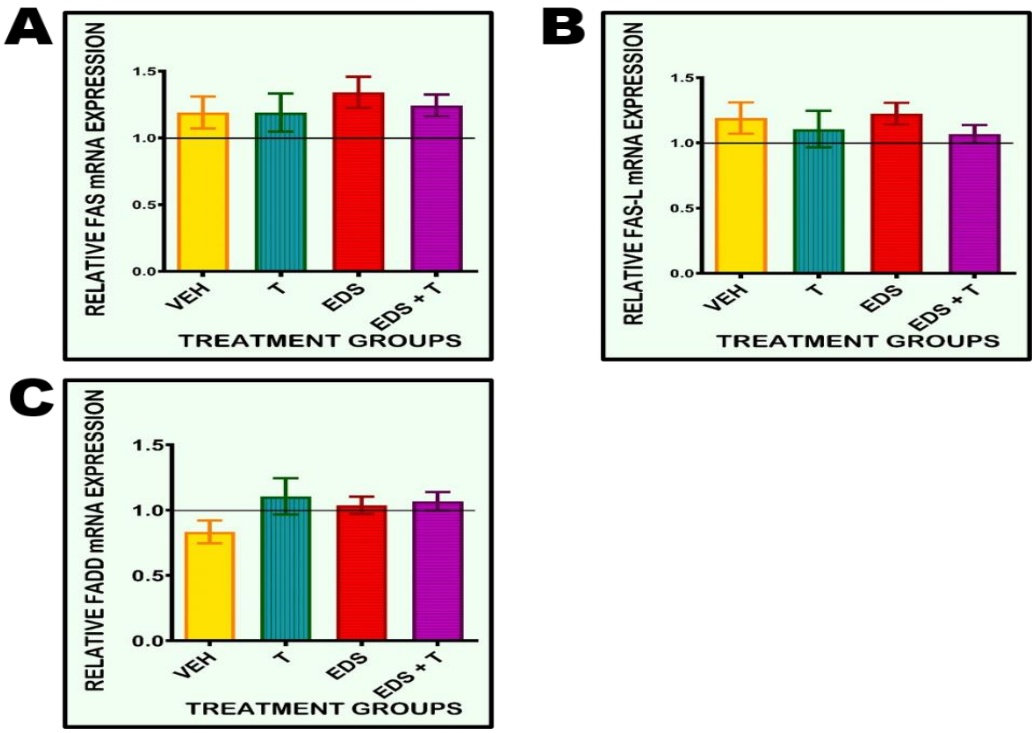
Note. (A) No treatment (NT); (B) EDS treated; (C) Testosterone replaced (EDS+T). Leydig cells in the interstitium are shown by red arrows. Arrows indicate the interstitium devoid of any Leydig cells. Scale bar is 100 µm.

Testosterone Withdrawal does not Affect Extrinsic Pathway of Apoptosis.

Apoptosis stimulating fragment (Fas/Fas-L) signaling is one of the major apoptotic pathways. The potential change in Fas and Fas-L gene expression post EDS treatment was determined by RT-qPCR. There were no significant changes ($p > 0.05$) in gene expression of Fas (see Figure 39 A), on Fas-L (see Figure 39 B), and Fas associated protein with death domain (FADD; see Figure 39 C) among all treatment groups.

Figure 39

Testosterone withdrawal results in no significant changes in gene expression of Fas, FasL and FADD indicating EDS treatment does not affect extrinsic pathway of apoptosis.



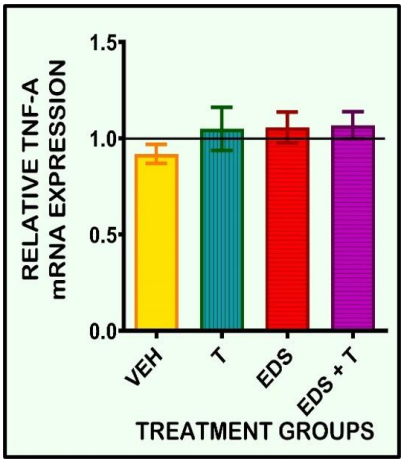
Note. (A) The expression of Fas relative to Gapdh as the reference gene. (B) The expression of FasL relative to Gapdh as the reference gene. (C) The expression of FADD relative to Gapdh as the reference gene. The horizontal line is the normalized value for the Not Treated (NT) rats. Error bars represent SEM with $n = 6$.

Testosterone withdrawal does not alter gene expression of proinflammatory apoptotic marker gene Tumor Necrosis Factor α (TNF-α).

To test if testosterone withdrawal alters pro-inflammatory cytokines, gene expression of proinflammatory apoptotic marker gene Tumor Necrosis Factor α (TNF-α) was determined. There were no significant changes ($p > 0.05$) in gene expression of TNF-α mRNA expression in adult rat testis (see Figure 40) among all treatment groups.

Figure 40

Testosterone withdrawal does not alter gene expression of the pro-inflammatory apoptotic marker gene TNF-α relative to Gapdh as the reference gene.



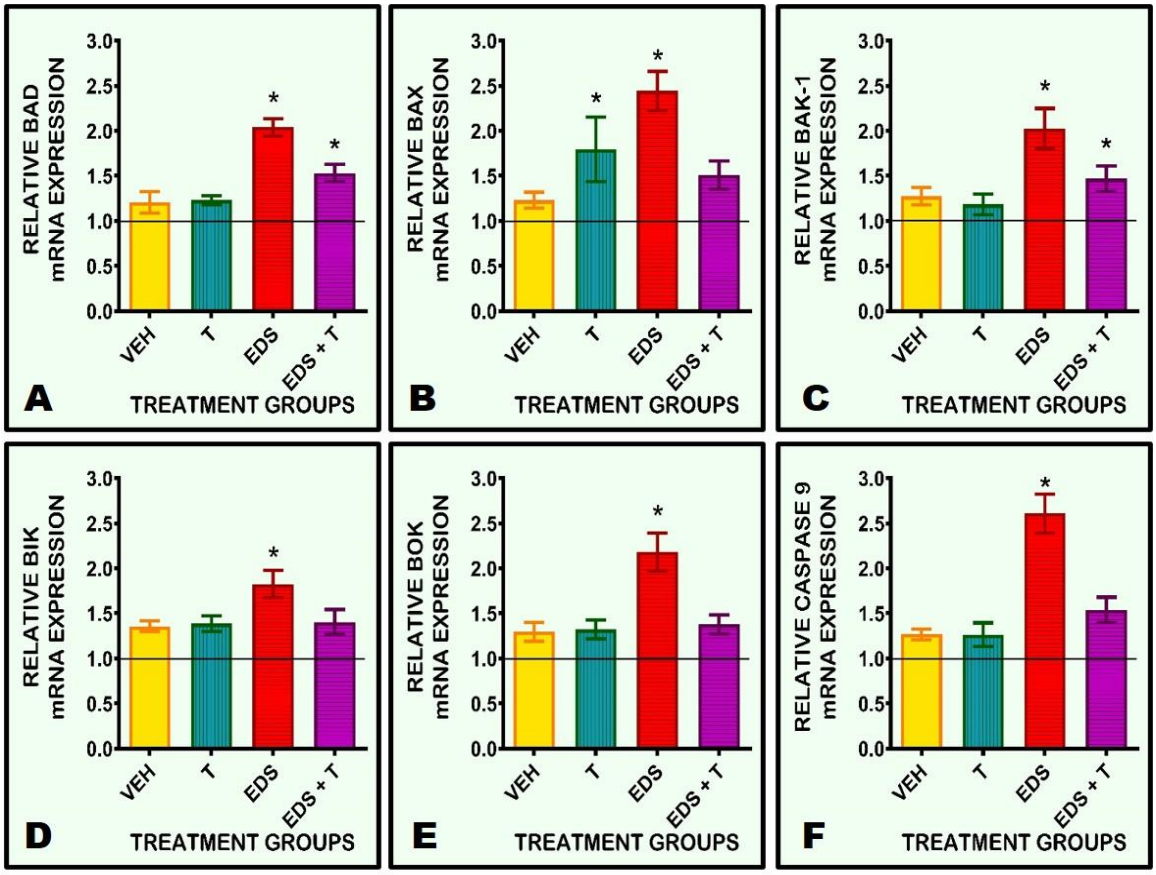
Note. The horizontal line is the normalized value for the no treatment (NT) rats. Error bars represent SEM with $n = 6$.

Testosterone Withdrawal Significantly Changes Gene Expression of Bcl2 Family of Proteins Involved in Intrinsic Pathway of Apoptosis

The Bcl-2 family of proteins involved in the intrinsic pathway is a protein family consisting of both anti-apoptotic (preventing apoptosis) and pro-apoptotic (triggering apoptosis) members. To investigate the underlying mechanism of how germ cell apoptosis occurs upon testosterone withdrawal, gene expression analysis was done for the key regulators of the intrinsic pathway of apoptosis using specific primers. Out of the wide variety of Bcl-2 proteins, several were analyzed. All the pro-apoptotic gene expression was significantly increased with testosterone withdrawal when compared to NT and vehicle treated groups (see Figure 41 A-F). Interestingly, we also observed a significant increase of anti-apoptotic gene expression upon testosterone loss (see Figure 42 A-D). On plotting a cumulative graph of all pro and anti-apoptotic genes, it was evident that the pro-apoptotic genes displayed a significantly greater increase in gene expression than the anti-apoptotic genes upon testosterone depletion. Thus, the pro-apoptotic genes may have facilitated germ cell apoptosis in EDS treated rat testis (see Figure 43). Composite of pro and anti-apoptotic genes suggesting that overall, pro-apoptotic genes rose to higher values in germ cells with testosterone loss.

Figure 41

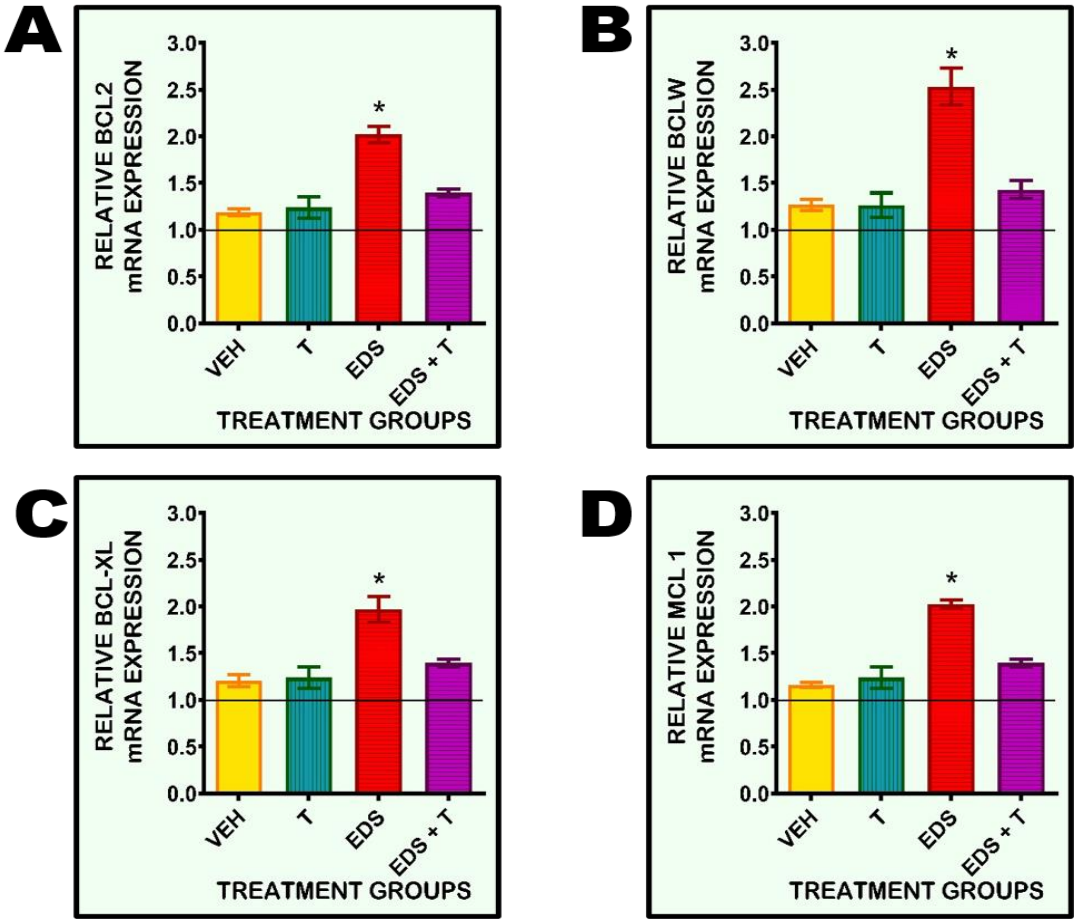
Testosterone withdrawal significantly increase gene expression of pro-apoptotic Bcl2 family of genes involved in intrinsic pathway of apoptosis.



Note. Gene expression of pro-apoptotic regulators of Bcl2 family relative to Gapdh as the reference gene. (A) BAD – BCL-2 Associated agonist of cell Death; (B) BAX - BCL-2 Associated X; (C) BAK-1 - BCL-2 Antagonist / Killer-1; (D) BIK - BCL-2 Interacting Killer; (E) BOK - Bcl-2-Related Ovarian Killer; (F) Caspase 9 - Apoptosis-Related Cysteine Protease. The horizontal line is the normalized value for the Not Treated (NT) rats. Error bars represent SEM with $n = 6$.

Figure 42

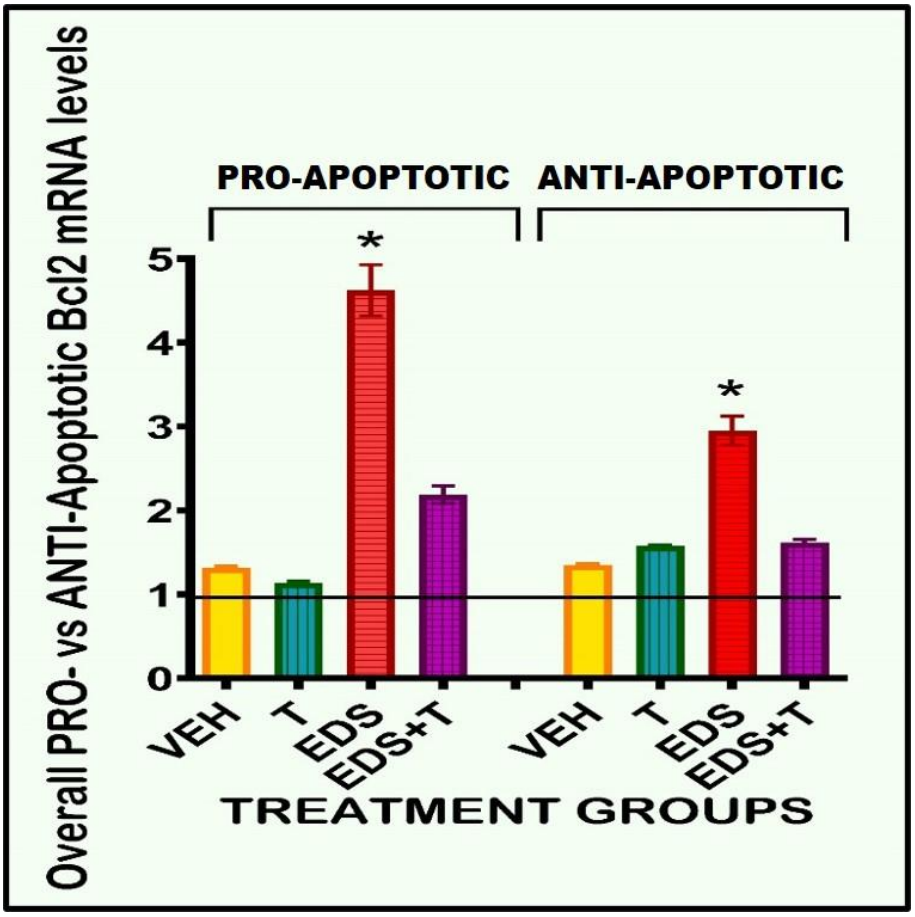
Testosterone withdrawal significantly increased gene expression of the anti-apoptotic Bcl2 family of genes involved in the intrinsic pathway of apoptosis.



Note. Gene expression of pro-apoptotic regulators of Bcl2 family relative to Gapdh as the reference gene. (A) BCL2 – B-cell lymphoma 2; (B) BCLW - Bcl-2-Like Protein 2; (C) BCL-XL - B-cell lymphoma extra large; (D) MCL 1 - Induced myeloid leukemia cell differentiation protein. The horizontal line is the normalized value for the no treatment (NT) rats. Error bars represent SEM with $n = 6$.

Figure 43

Testosterone withdrawal significantly increase gene expression of pro-apoptotic genes more than the expressions of anti-apoptotic genes of the Bcl2 family of proteins involved in the intrinsic pathway of apoptosis.



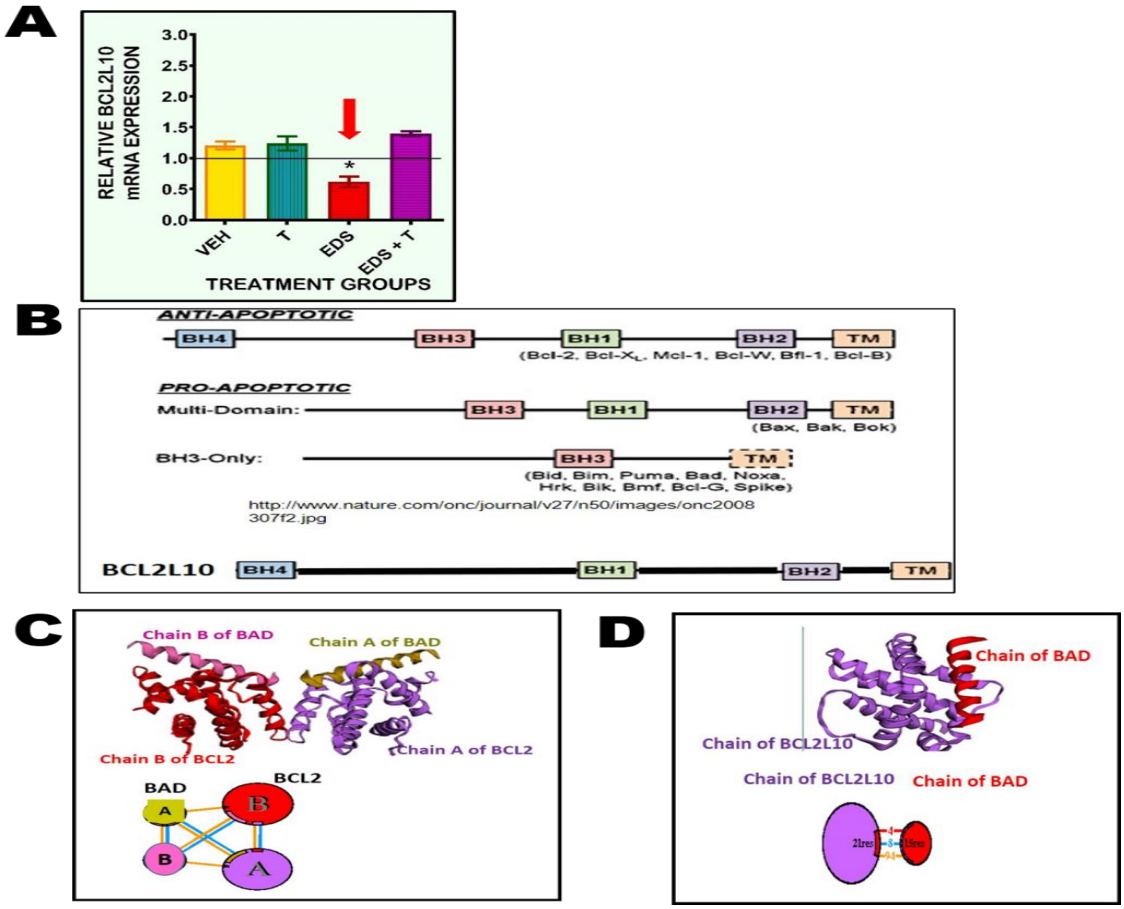
Note. The horizontal line is the normalized value for the Not Treated (NT) rats. Error bars represent SEM with $n = 6$. Single asterisk (*) indicates significant difference from NT and VEH in their own groups.

Presence of BH3 Domain in BCL2 Proteins is Crucial for Performing Anti-Apoptotic Activities

Interestingly, only one gene had reduced gene expression upon testosterone withdrawal, BCL2L10 (see Figure 44 A). This is an anti-apoptotic protein that is proposed to bind to pro-apoptotic proteins to inhibit apoptosis. To investigate this difference further, the protein domains of BCL2L10 were compared to the other BCL2 proteins. The BH3 domain was absent in BCL2L10 protein which was present in all other BCL2 proteins (see Figure 44 B). Protein-protein interaction using JPred revealed that BCL2 proteins with a BH3 domain would covalently bind to each other (see Figure 44 C), but absence of BH3 domain in BCL2L10 causes it to bind with other BCL2 proteins using hydrogen bonds (see Figure 44 D). From our research findings, our predicted model was that the proteins with the BH3 domain can form heterodimers with each other that is crucial for the apoptotic pathway. This contrasts with pro-apoptotic protein interaction with BCL2L10 inhibiting heterodimer formation.

Figure 44

Presence of the BH3 domain in BCL2 proteins is crucial for performing anti-apoptotic activities.



Note. (A) Gene expression of BCL2L10 using Gapdh as the reference gene. Error bars represent SEM with $n = 6$. The horizontal line represents the value of BCL2L10 for Not Treated (NT) rats normalized to 1.0. A single asterisk (*) indicates significant difference from VEH, T & EDS+T. (B) List of BCL2 proteins with their domains. (C) Predicted protein-protein interaction model for BAD (pro-apoptotic protein) and BCL2 (anti-

apoptotic protein), both containing BH3 domains.; (D) Predicted protein-protein interaction model for BAD (pro-apoptotic protein) containing BH3 domain and BCL2L10 (anti-apoptotic protein in which BH3 domains is absent).

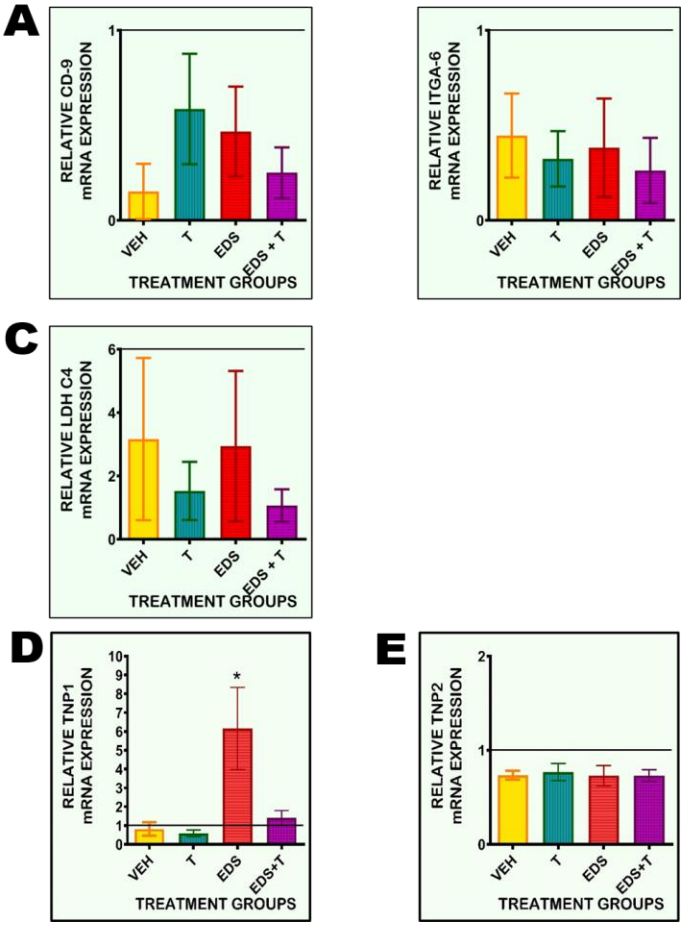
Is Testosterone Dependent Germ Cell Apoptosis Targeted Towards a Particular Cell Population?

Ablation of Testosterone Causes Alterations in Specific Germ Cell Populations

Different germ cells produce unique proteins during their development that enables them to be identified. Undifferentiated spermatogonial cells commonly express surface protein markers α-6-integrin and CD9 (tetraspin transmembrane protein). Change in the level of spermatogonia with all treatments were observed with alterations in gene expression of α-6-integrin and CD9 (see Figure 45 A, B). LDH-C4 gene is expressed exclusively during meiosis in spermatocytes. LDH-C4 gene expression increased with testosterone ablation (see Figure 45 C). Transition Protein (Tnp) 1 and 2 are proteins that are involved in the packaging of sperm nuclear DNA during spermiogenesis. They take the place of histones associated with the sperm DNA, which are replaced by protamines. Round spermatids produce and store mRNAs for transition protein. Tnp 1 is a round spermatid unique protein marker. Expression of Tnp 1 significantly increased with loss of testosterone (see Figure 45 D). Tnp 2 expressions did not change in elongated spermatids (see Figure 45 E).

Figure 45

Ablation of testosterone causes alterations in specific germ cell population.



Note. (A) Gene expression of CD9 using Gapdh as the reference gene. (B) Gene expression of α-6-integrin using Gapdh as the reference gene. (C) Gene expression of LDHC4 using Gapdh as the reference gene. (D) Gene expression of Tnp 1 using Gapdh as the reference gene. (E) Gene expression of Tnp 2 using Gapdh as the reference gene. Error bars represent SEM with $n = 6$. The horizontal line represents non treated (NT) rats normalized to 1.0. (*) indicates a significant difference from NT.

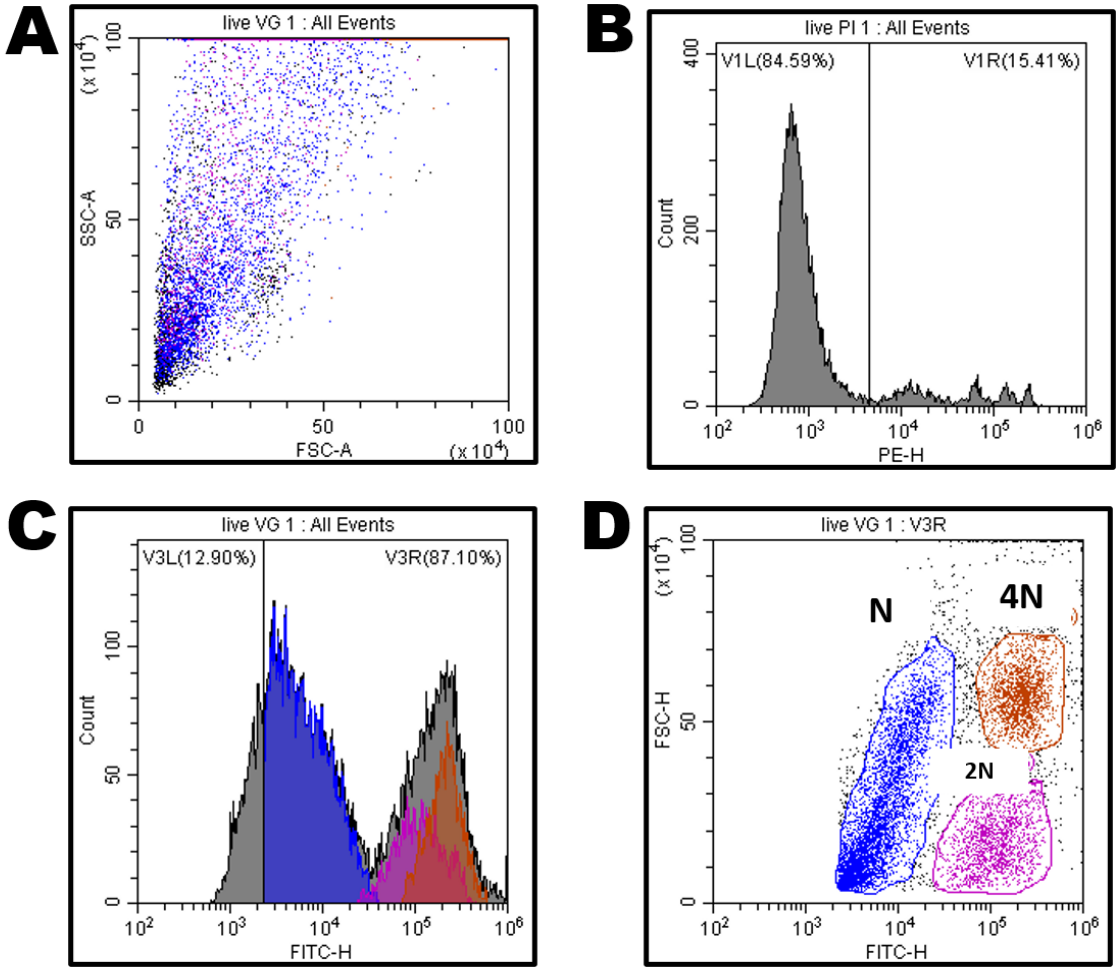
Testosterone Withdrawal Impacts Cell Death of Specific Germ Cell Populations

Identification and cell death percentage of germ cell subpopulation was analyzed by modifying the protocol from Petit et al. (1995), after DNA staining by Vybrant Green (VG) a dye that bind stoichiometrically to DNA (see Figure 46 C) and dead cell indicator dye propidium iodide (PI; see Figure 46 B). The use of VG binding specifically and stoichiometrically to haploid (N), diploid (2N), and tetraploid (4N) permitted a good separation of germ cell subpopulations (see Figure 46 D). The haploid region (N) was easily identified using morphological evolution of the nucleus that corresponds to the different steps in maturation from the round to the elongated spermatids. The 2N cells were identified as spermatogonia due to their morphology. In the tetraploid region cells were similar diameter to 2N cells but the chromatin was uncondensed. These include primary spermatocytes, pachytene, leptotene, and zygotene spermatocytes (Petit et al. 1995). Testosterone loss by EDS induces significant germ cell death, significant decrease in N and decrease in 2N germ cell populations.

Isolated germ cells stained with PI, used to detect dead cells, was interpreted as a significant increase in germ cell loss in testis upon testosterone withdrawal (see Figure 47 A). This loss was reversed when testosterone was replaced. Forward scatter and side scatter data revealed the presence of three distinct cell populations according to cell size and granularity (see Figure 46 D). Further gating revealed a significantly lower N germ cell count post-EDS treatment (see Figure 47 B). 2N germ cell number was reduced post EDS treatment (see Figure 47 C) but there was no change in 4N germ cell number.

Figure 46

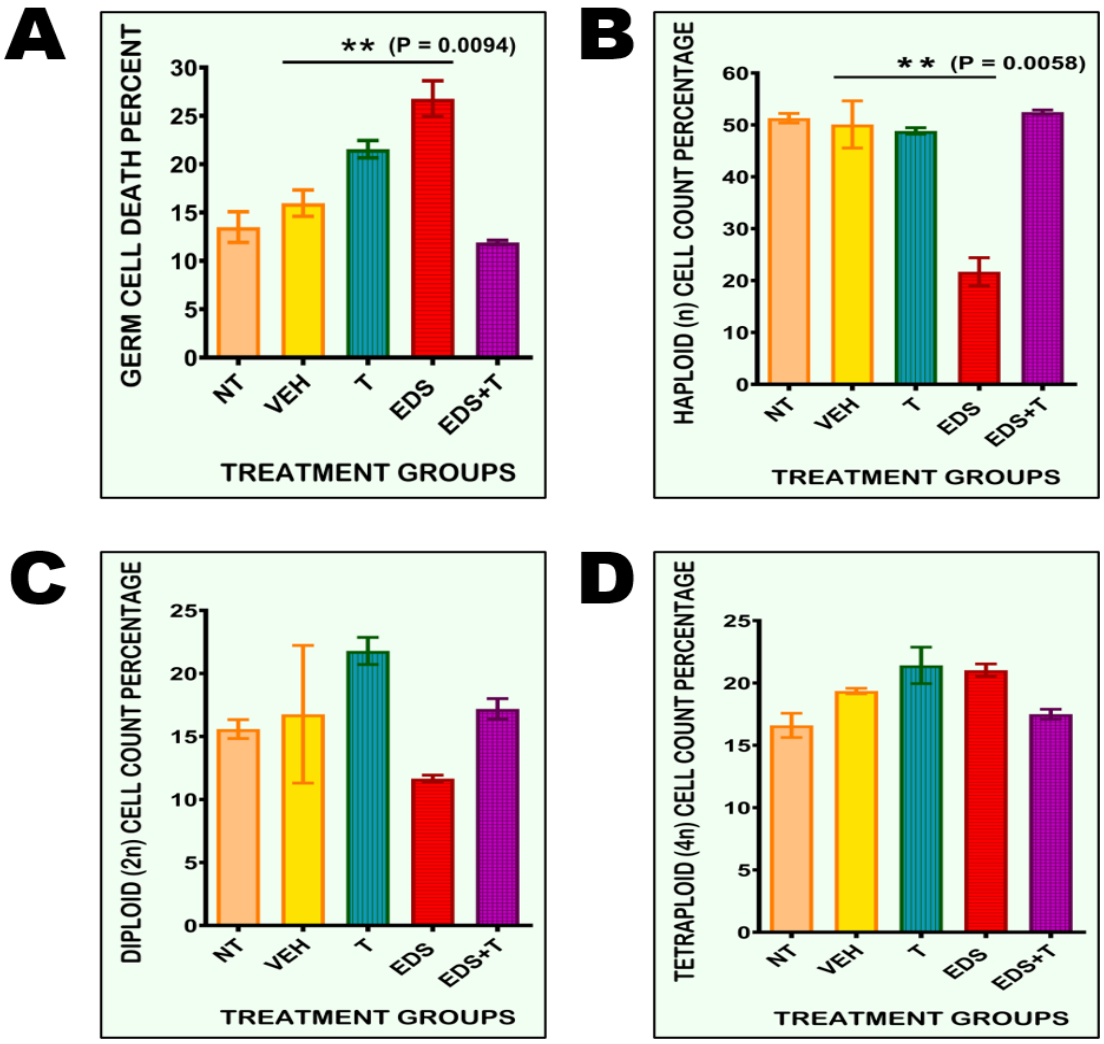
Representative images from multiparameter flow cytometric analysis of at least 10, 000 rat testes NT germ cell suspensions respectively with DNA staining dye VG binding stoichiometrically to DNA and dead cell indicator dye PI.



Note. (A) FSC (Forward scatter) and SSC (Side scatter). (B) Dead cell Percentage by PI staining (C) DNA staining by VG (D) Gated cell populations specific to VG binding to DNA.

Figure 47

Testosterone loss by EDS induces significant germ cell death and a significant decrease in N germ cell populations.



Note. (A) Percentage of germ cell death (B) Percentage of N cells (C) Percentage of 2N cell (D) Percentage of 4N cell among all treatment groups. Error bars represent SEM with $n = 3$. (*) indicates significant difference from NT.

How Does Testosterone Impact the Alternate Pathway, if any, of Testosterone Action in Germ Cells?

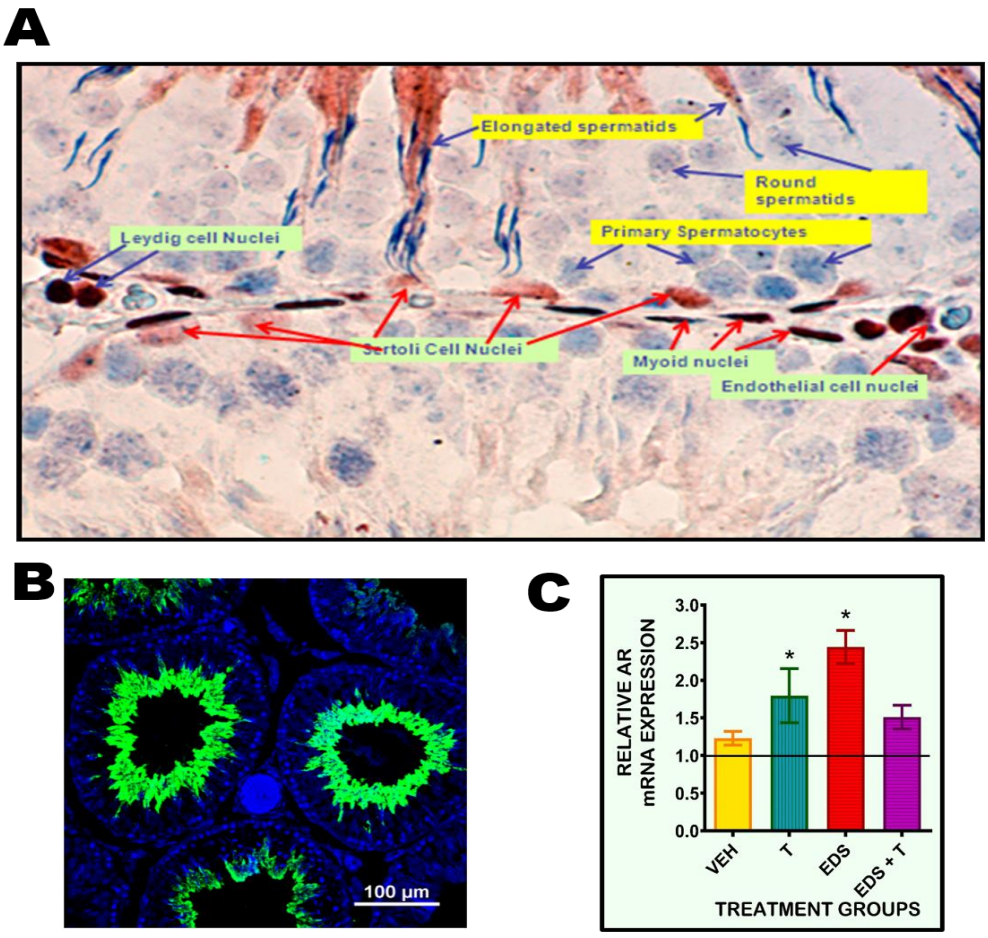
Androgen Receptors are Present in the Nuclei of Somatic Cells in Testis

Testosterone, being a steroid molecule, is known to act via the classical canonical pathway. That is, testosterone diffuses through the plasma membrane and binds to the androgen receptors bound to heat shock proteins in the cytoplasm. Binding leads to androgen receptor dimerization causing alteration in the conformation of androgen receptors initiating receptor release from the heat shock proteins. Dimerized androgen receptors along with testosterone translocate into the nucleus where it binds to specific DNA sequences called ARE. Upon binding, it allows recruitment of co-activators and co-repressors that alter expression of genes to change cellular functions.

Based upon this knowledge, it is crucial to investigate which cells possess androgen receptors. Nova red precipitated targeted immune staining localized Nuclear androgen receptors (nAR) present in nuclei of Sertoli cells, Leydig cells, myoid cells, endothelial cells, and in stripped cytoplasm of elongated spermatids but neither in mitotic nor meiotic germ cells (see Figure 48 A - B). Messenger RNA expression of androgen receptors was altered with changes in testosterone levels and this suggested that testosterone regulates AR expression; however, the exact mechanism remains unknown and requires future investigation (see Figure 48 C).

Figure 48

Androgen receptors are present in the nuclei of somatic cells in testis.



Note. (A) Cross section of seminiferous tubule of rat testis stained with Nova-red, a dye staining androgen receptor. (B) Fluorescent microscopy of anti-AR antibody-stained cross section of seminiferous tubule. (C) Gene expression of nuclear androgen receptors relative to Gapdh as the reference gene. The horizontal line is the normalized value for the NT rats. Error bars represent SEM with $n = 6$. (*) indicates a significant difference from NT.

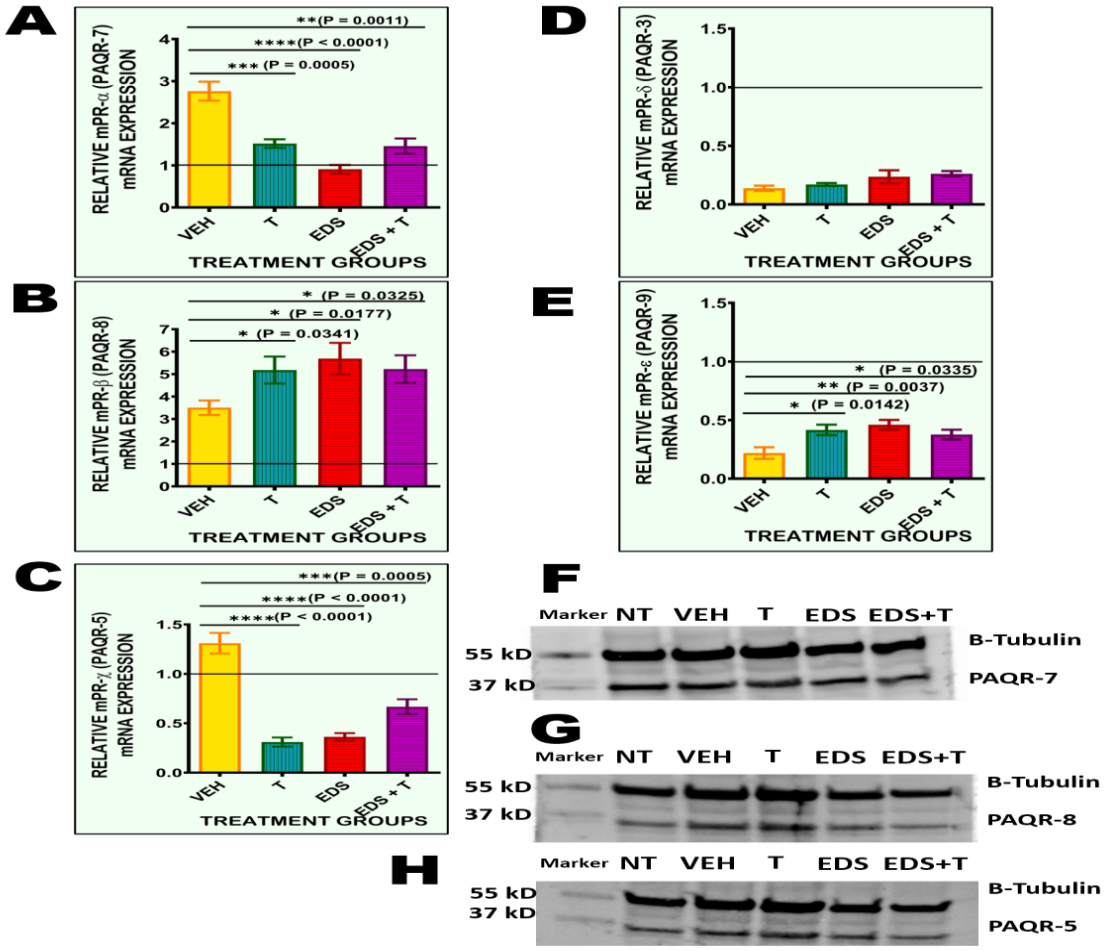
Membrane Progesterone Receptors Serve as an Alternate Pathway of Testosterone Action in Germ Cells

Membrane progesterone receptors (mPRs) are a group of 7 transmembrane domain cell surface receptors belonging to the progestin and adipoQ receptor (PAQR) family that bind to circulating progesterone & acts like a G protein coupled receptor. mPRs act via non-genomic signaling pathways. The reason for choosing progesterone pathways is the presence of similar a A-ring structure in testosterone and progesterone, both of which are known to bind with their receptors mainly via A-ring.

mPRs are classified into five groups known as mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6), and mPRϵ (PAQR9). mPR-alpha (mPRα) is a protein that in humans is encoded by the *PAQR7* gene. It is a steroid receptor which binds progesterone *in vitro*. mPR-beta (mPRβ) is a protein that in humans is encoded by the *PAQR8* gene. mPR-gamma (mPRγ) is a protein that in humans is encoded by the *PAQR5* gene. mPR-delta (mPRδ) is a protein that in humans is encoded by the *PAQR6* gene. mPR-epsilon (mPRϵ) is a protein that in humans is encoded by the *PAQR9* gene. RT-qPCR data supports the presence in testis of three main mPRs. According to gene expression using specific primers, these three seemed to be regulated by testosterone (see Figure 49 A - C). mPRδ and mPRϵ were scarcely found in testis (see Figure 49 D, E) but is in abundance in brain cells. Western Blot analysis of mPR proteins were abundant in testicular tissues but changes in testosterone concentration among the treatments (see Figure 49 F – H).

Figure 49

Membrane progesterone receptors serve as an alternate pathway of testosterone action in germ cells.



Note. Gene expression of (A) mPRα, (B) mPRβ, (C) mPRγ, (D) mPRδ and (E) mPRϵ relative to Gapdh as the reference gene. The horizontal line is the normalized value for the NT rats. Error bars represent SEM with *n* = 6. (*) indicates significant difference from NT. Western blot analysis of mPRα (F), mPRβ (G), mPRγ (H) relative to β-tubulin as the reference gene.

Testosterone Competitively Binds to Membrane Progesterone Receptors on Germ Cells

To confirm whether testosterone is specifically binding with mPRs and is able to maintain binding in presence of a low level of progesterone and extrinsic testosterone, competitive binding assays were performed. Anti-mPR-α antibodies tagged with alexa fluor 647 (red fluorescence) was used to tag membrane progesterone receptor- α, whereas testosterone and progesterone was tagged with FITC (green fluorescence). BSA coupled with testosterone and progesterone prevented these hormones from entering germ cells through the membrane.

Colocalization studies on germ cells isolated from normal rat testis had presence of mPR-α receptors on the membrane (see Figure 50 a-e) and progesterone-BSA-FITC (see Figure 50 f-j) and testosterone-BSA-FITC (see Figure 50 k-o) could successfully and distinctly bind to the membrane receptors. (see Figures 53, 54). When each of the above-mentioned situations were incubated with extrinsic cold testosterone, binding of progesterone on mPRs became fuzzy and indistinct (see Figures 50 p-t, 53, 54). But testosterone binding maintained the distinct rim structure upon binding (see Figure 50 u-y).

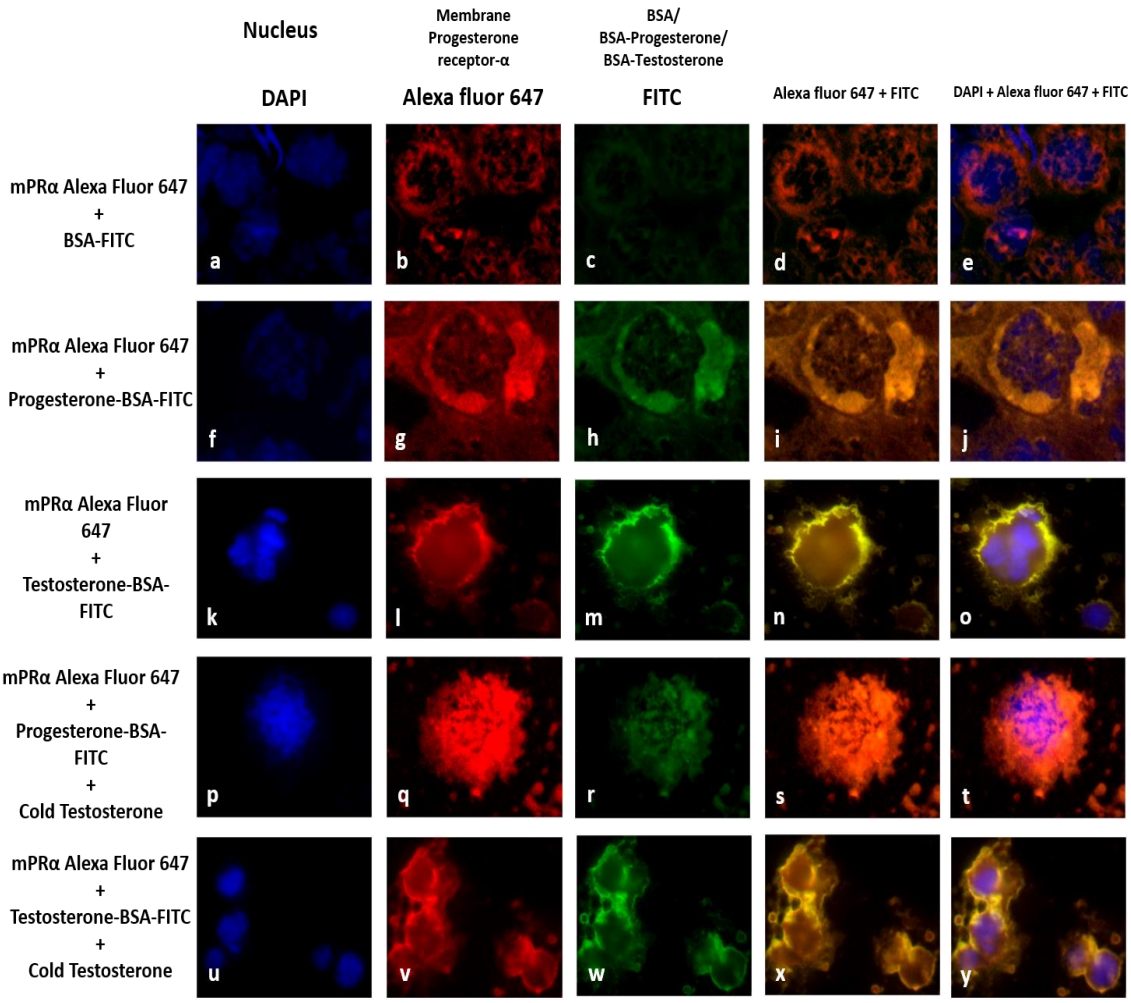
When cells isolated from 10-day post-EDS treated tissues were used, that is, when testosterone was absent in testis, expression of mPRs was reduced and there appeared to be significantly reduced binding of testosterone or progesterone on mPRs (see Figures 51

f-o, 53, 54) this binding pattern was significantly reduced upon cold testosterone treatment (see Figures 51 p-y, 53, 54).

This reduced expression and colocalization reverted to a normal binding pattern with testosterone replacement post-EDS treatment (see Figures 52, 53, 54). Testosterone binding was significantly reduced in EDS-treated germ cells (see Figures 53, 54).

Figure 50

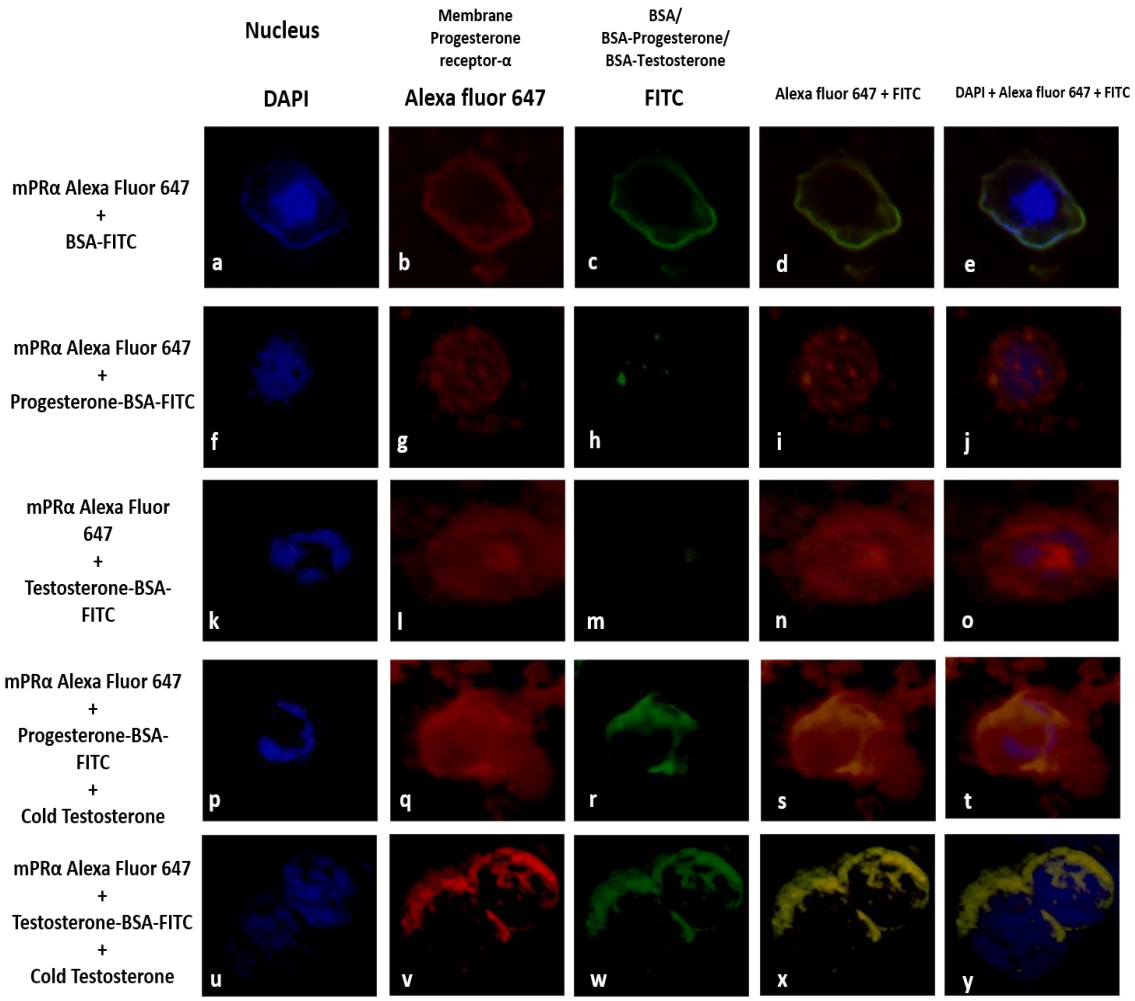
Competitive binding assay of NT rat testis germ cells.



Note. (a-e) Germ cells tagged for mPR-α and BSA-FITC; (f-j) Germ cells tagged for mPR-α and progesterone-BSA-FITC; (k-o) Germ cells tagged for mPR-α and testosterone-BSA-FITC; (p-t) Germ cells tagged for mPR-α and progesterone-BSA-FITC and competitively incubated with cold testosterone; (u-y) Germ cells tagged for mPR-α and progesterone-BSA-FITC and competitively incubated with cold testosterone.

Figure 51

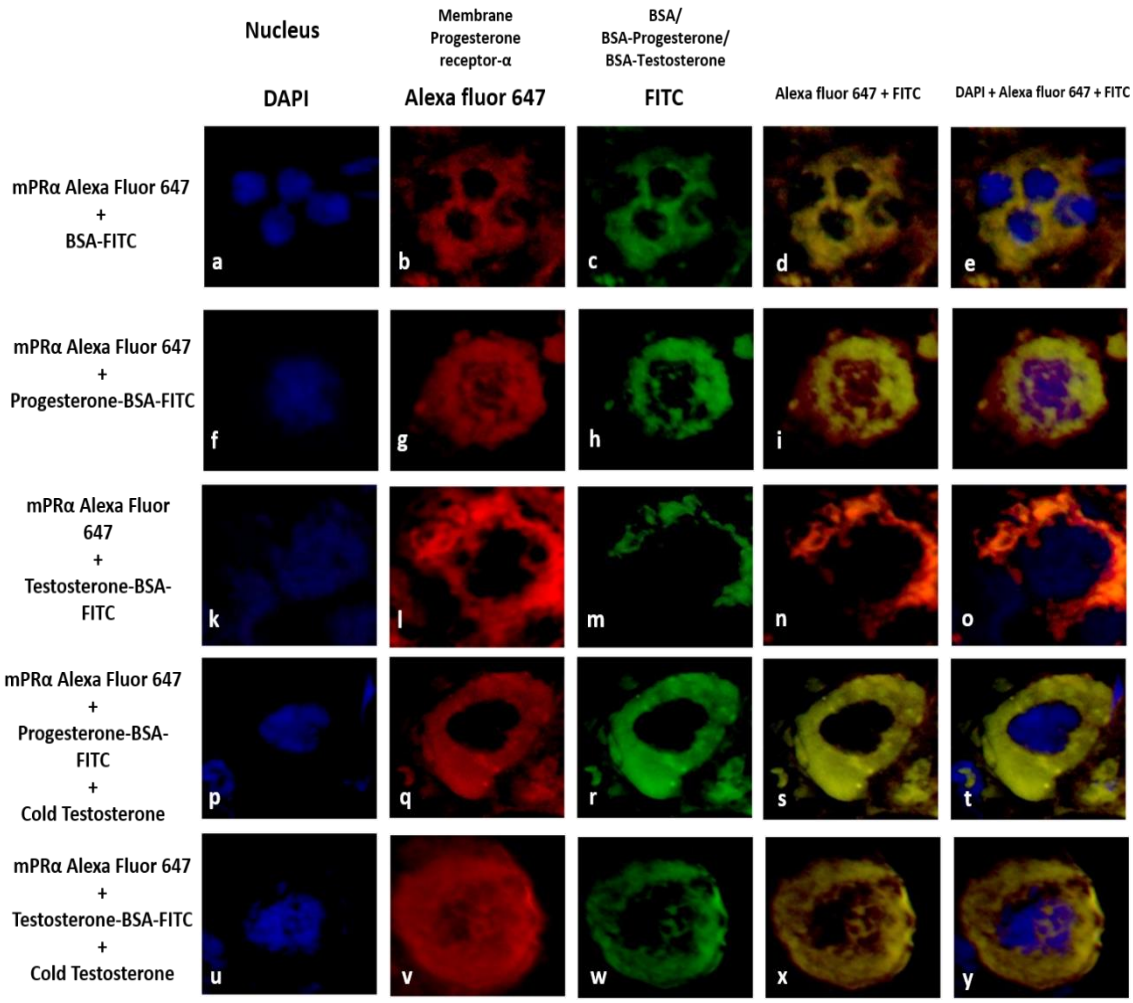
Competitive binding assay of EDS rat testis germ cells



Note. (a-e) Germ cells tagged for mPR-α and BSA-FITC; (f-j) Germ cells tagged for mPR-α and progesterone-BSA-FITC; (k-o) Germ cells tagged for mPR-α and testosterone-BSA-FITC; (p-t) Germ cells tagged for mPR-α and progesterone-BSA-FITC and competitively incubated with cold testosterone; (u-y) Germ cells tagged for mPR-α and progesterone-BSA-FITC and competitively incubated with cold testosterone.

Figure 52

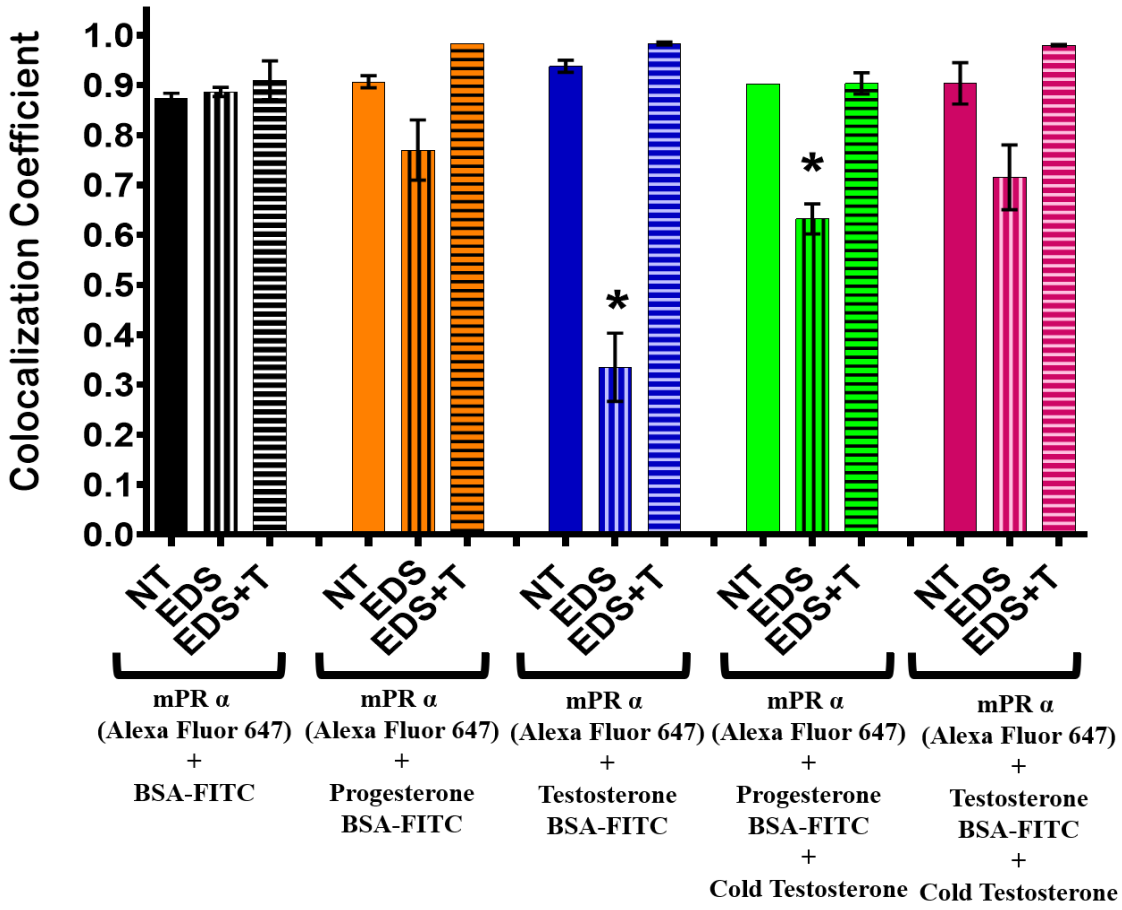
Competitive binding assay of EDS+T rat testis germ cells



Note. (a-e) Germ cells tagged for mPR-α and BSA-FITC; (f-j) Germ cells tagged for mPR-α and progesterone-BSA-FITC; (k-o) Germ cells tagged for mPR-α and testosterone-BSA-FITC; (p-t) Germ cells tagged for mPR-α and progesterone-BSA-FITC and competitively incubated with cold testosterone; (u-y) Germ cells tagged for mPR-α and progesterone-BSA-FITC and competitively incubated with cold testosterone.

Figure 53

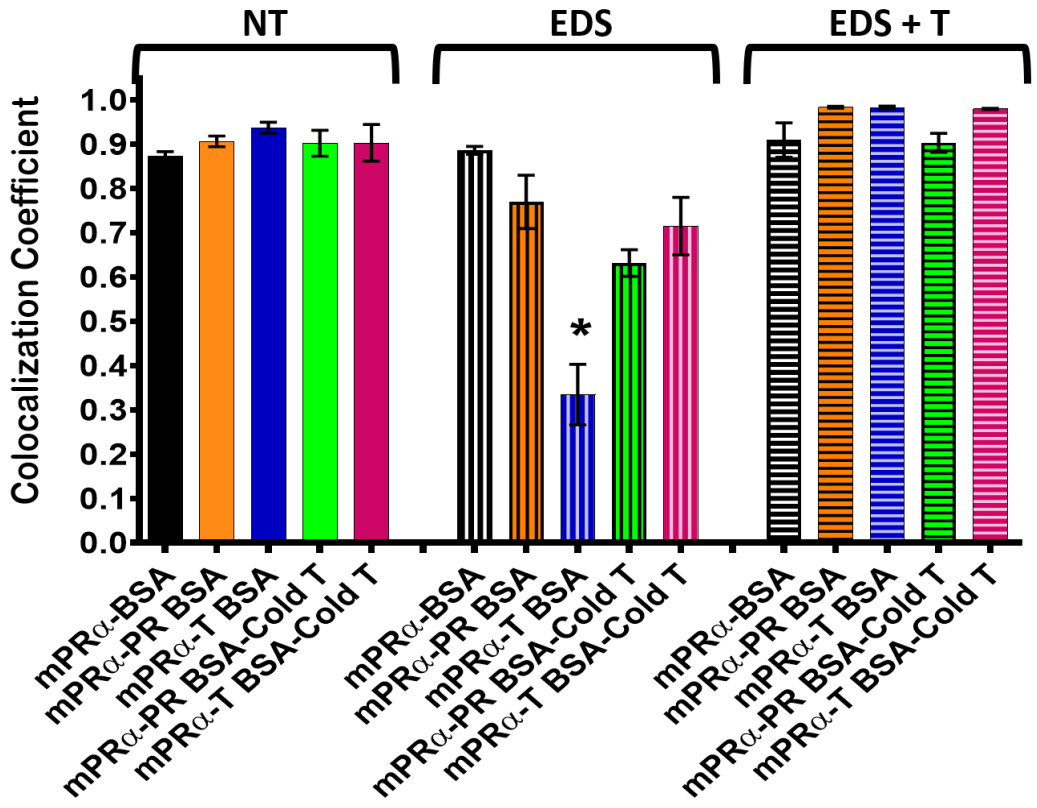
Testosterone and progesterone shows significantly reduced binding to mPR-α in EDS treated rat testis germ cells when compared to NT and EDS+T.



Note. (*) indicates a significant difference from NT and EDS+T. *n* = 85 to 100 cells.

Figure 54

Testosterone only shows significantly reduced binding to mPR-α when compared to all binding assays in EDS treated rat testis germ cells.



Note. (*) indicates significant difference from various binding assays within EDS treated rat testis germ cells. *n* = 85 to 100 cells.

CHAPTER IV

DISCUSSION

We investigated the role of testosterone on testicular germ cell viability and germ cell apoptosis 10 days post EDS treatment. The focus of our study was to see whether testosterone is responsible in maintaining spermatogenesis or loss of testosterone targets the loss of a specific type of germ cells. So, to compensate the loss of testosterone, we investigated the presence of an alternate testosterone action pathway on germ cells.

The methane sulfonic ester of ethylene glycol, ethane1,2-dimethanesulfonate (EDS) is a unique testicular toxicant. A single dose of EDS destroys the Leydig cell population that is reported to recover at ~60 days after EDS treatment. The Leydig cells are thought to regenerate from mesenchymal (undifferentiated) fibroblast-like precursors (Jackson AE et al 1986a; Jackson NC et al 1986b; Kerr JB et al 1986; Morris ID et al 1986; Morris ID 1996). EDS treatment of rats leads to alterations in the cytoplasm and nuclei of the Leydig cells including vesiculation in the smooth endoplasmic reticulum, focal hypertrophy of the Golgi apparatus and clumping of the nuclear chromatin (Kerr JB et al 1986; Morris ID et al 1986). Prolonged exposure on the Leydig cells exhibit further degenerate alterations leading to their disappearance through the phagocytic activity of macrophages (Jackson NC et al 1986B; Kerr JB et al 1985; Morris ID et al 1986). These morphological changes are reminiscent of those described for apoptosis in other cell systems (Earnshaw WC 1995; Kroemer G et al 1995; Wyllie AH 1980), suggesting that

Leydig cells undergo programmed cell death from an apoptotic pathway in response to external stimuli. Testis sections from rats treated in vivo with EDS have inter-nucleosomal DNA cleavage 24 hr after treatment (Henriksen et al 1995). Increased DNA fragmentation was found in crude interstitial cell preparations obtained from rats after hypophysectomy (Tapanainen et al 1993) and indicates that Leydig cell apoptosis may be a more general phenomenon.

Testicular weights of testosterone depleted rats following EDS treatment have a significant and progressive weight loss or reduction in comparison to rats with normal testosterone levels. In the testosterone replacement group (EDS + T), the testicular weights were comparable to normal testicular weights and vehicle treated-control testis. Loss of Leydig cells that constitute 3-4 % of testicular volume/weight does not account for the testicular weight loss following EDS treatment. However, the EDS treatment does not have a large effect directly upon the germ cell population, since EDS is given only once at day '0' and there is significant increase in germ cell apoptosis by days 5 and 7 post-EDS treatment. However, germ cells losses via apoptosis have a prominent role in testicular changes (weight and volume) following EDS, 5 through 20 days post-EDS (Dutta, D et al 2019).

Testosterone loss due to Leydig cell ablation in the first 2 days after EDS treatment causes a reduction of testicular size due to increased germ cell apoptosis (Dutta, D et al 2017) , and possibly a decreased testicular fluid secretion - a process thought to be regulated by estrogen and indirectly by testosterone since testosterone is the immediate

precursor/substrate for estrogen synthesis by aromatase that can be found in the somatic cells of the gonads (Sharpe, et al 1990; Sowerbutts, et al 1986). Therefore, testosterone is required for maintanance of normal testicular weight.

Insulin-like Protein 3/relaxin-like factor (Insl3/RLF) is thought to be unique to gonadal development and maturation and is synthesized in Leydig cells in the male. In the female, theca interna cells of the developing follicle and the corpus luteum that develops after follicular rupture and ovum release also produce Insl3. (Ravender et al 2011). HSD3β2 is an isoform of HSD3β and is found primarily in testes, ovaries, and adrenals. After EDS treatment, mRNA loss of *Insl3* and *Hsd3β2* in the testis corresponded more closely with Leydig cell apoptosis and this supports the loss of most mature Leydig cells in the first 24 hours and the rapid depletion of serum testosterone to undetectable levels in rats, 7 and 10 days post-EDS. Loss of testosterone was accompanied with a significant reduction of testicular weight and a substantial increase in the germ cell apoptosis, consistent with the previous reports (Dutta, D et al 2019). Loss of primary spermatocytes and round spermatids via apoptosis would contribute to the testicular weight loss since some of the apoptotic cells would have been absorbed by the Sertoli cells (nurse cells) and others were found downstream in sperm granulomas of the epididymis of EDS-treated rats post 7 and 10 days of EDS treatment (Dutta, D et al 2019).

Ten days post-EDS, testosterone replacement (EDS + T), did not maintain the levels of *Insl3* and *3βHSD2* expression or help recover these levels and remained near

undetectable since Leydig cells were depleted earlier. However, in contrast to EDS-treated rats, VEH rats receiving 25% DMSO and sesame seed oil maintained testicular weights and viability of germ cells at control levels and this was also true for EDS treated rats receiving replacement of testosterone (EDS+T). Interestingly, the level for *Insl3* and *3βHSD2* were significantly lower in the testosterone supplemented group (T) compared to vehicle control (VEH). Gene and protein expressions of *Insl3* and *3βHSD2* in testis is directly regulated by LH secreted by pituitary gland (Ferlin et al 2006). Therefore, exogenous supplemental testosterone treatment (T) resulted in an elevated circulating testosterone (serum) that may have down-regulated the mRNA expression of *Insl3* through negative feedback to the hypothalamic-pituitary-gonad axis – reducing the release of LH from the anterior pituitary. Although normal germ cell viability and testicular weights were maintained with testosterone replacement after EDS, the testosterone have no protective effect on EDS elimination of the Leydig cells (Allan, et al 1992; Blanchard, et al. 1996; Krischel, et al 1999; Nakagawa, et al 1997; Richburg, 2000; Suschek, et al 1999; Shinoda, et al. 1999).

According to studies, apoptosis is the dependent on chemically induced testicular toxicity (Blanchard, et al. 1996; Brinkworth, et al. 1995; Lee, et al. 1997; Nakagawa, et al. 1997). Mechanisms activating apoptosis changes due to target components affected within the testis. Apoptotic pathways involving Bcl-2/Bax has been well documented (Desagher, et al 2000; Green, et al 1998) and these pathways play an important role in maintaining the development of spermatogenesis (Knudson, et al. 1995; Print, et al. 2000;

Rodriguez, et al. 1997). The pro-apoptotic player Bax and the anti-apoptotic protein Bcl-2 are key modulators and their relative levels determine the fate of cells (Butt, et al. 2000; Parker, et al. 2000; Yang, et al. 2001). Bax positively induces apoptosis resulting in depleting the growth factors while Bcl-2 inhibits apoptosis by binding to and inactivating multiple pro-apoptotic proteins (Youle, et al. 2008). The regulation of the expression levels of the two genes determines whether the cells will undergo apoptosis or not (Korsmeyer, 1999).

Apoptosis is an active cellular process of gene-directed self-destruction in that cells die in a controlled fashion either spontaneously or in response to various environmental stimuli and/or chemicals. Our study of the apoptotic germ cells after EDS treatment and using total RNA analysis for gene expression revealed that a significant increase in pro-apoptotic and anti-apoptotic genes were both expressed after loss of testosterone with EDS and testosterone replacement (see Figures 41, 42). Apoptotic germ cells seemed to be retained by an apparent balance of similar expressions of pro versus anti-apoptotic genes (see Figures 43). Many germ cells were not detected as apoptotic while many other germ cells gave a strong TUNEL (a late-stage marker of apoptosis) positive response (Dutta et al. 2019). Since both pro and anti-apoptotic genes were detected from total RNA isolated from testes, the shifts in pro-apoptotic and anti-apoptotic markers (genes) could have come from different germ cells or may have existed in the same apoptotic cells, since the gene expression levels varied mostly by one or two cycles by real time-PCR in all treatment groups. This mix of testicular cells would have

been difficult to decipher directly by immunohistochemistry since gene expression changes were not large. The apoptotic cells were revealed by TUNEL at 5 and 7-days post-EDS, while EDS with testosterone replacement did not have a significant increase in apoptotic germ cells (by TUNEL) at 5 or 7 days (Dutta et al. 2019). Isolated cell populations from treated testes at 10 days post-EDS, and analysis with PI staining detected dead cells with flow cytometry. The dead cells were primarily haploid cells i.e. round spermatids, that had a significant number of dead cells. Thus, with EDS alone and TUNEL staining at 5 and 7 days (Dutta et al. 2019) and with PI staining at 10-days to detect dead cells, this evidence strongly suggested that the germ cell apoptosis with EDS was due to loss of testosterone after Leydig cell depletion. After EDS + testosterone replacement, the apoptotic cell numbers were like control groups, again suggesting that testosterone maintenance after EDS prevented the haploid gamete cell loss (Chittenden, et al. 1995; Sattler, et al. 1997).

On the basis of function and domain structure, Bcl2 genes are of two types: first, proteins that act as inhibitors of apoptosis harbor at least three BH domains-BH1, BH2, and BH3 and second, a transmembrane domain. The minimum requirement for a death-promoting protein is a BH3 domain. The BH3 domain is a stretch of 16 amino acids in Bak that is required to form heterodimeric complexes with anti-apoptotic members of the Bcl-2 family and to promote apoptosis. We found that all the genes containing BH3 domains responded with changing levels of testosterone and was significantly increased with loss of testosterone.

To maintain mitochondrial homeostasis and integrity, Bcl-2 proteins form ion channels. BH3-domain-containing proteins bind to a hydrophobic pocket of the anti-apoptotic protein to antagonize their function by creating a nonfunctional or dysfunctional pore compromising mitochondrial integrity. A dysfunctional channel could alter ion-selectivity, rendering it pro-apoptotic. Hetero-dimerization then leads to conformational changes of these proteins so they cannot be inserted into mitochondrial membranes. (Kelekar, et al. 1997). We analyzed genes with and without a BH3 domain. The gene without BH3 domain, Bcl2L10, had a reduced gene expression level with loss of testosterone which is completely opposite of the data in BH3 containing genes. Therefore, it can be concluded that the BH3 domain is needed for proper functioning of apoptotic genes in rat testicular germ cells.

Mammalian spermatogenesis is a cascade of biochemical events with morphological alterations involving undifferentiated diploid stem cell, the primitive type A spermatogonium, and highly differentiated haploid cell, the spermatozoon. These cells are differentiated into three phases: spermatogonial proliferation and renewal, mitosis and meiosis, and spermiogenesis (Meistrich, 1977). In adult male germ cells, proliferation and differentiation associated parameters were detected by flow cytometry and include DNA content (Mc Lean-Grogan et al. 1981; Pinkel et al. 1979), ploidy and chromatin condensation (Meistrich et al. 1978), size (Spanb et al. 1984), shape (Benaron et al. 1982), RNA content (Evenson et al. 1983), exchange of histones for sperm-specific transition proteins (S), and sperm-associated immunoglobulins (Haas et al. 1984).

Changes in testicular mitochondria during spermatogenesis like mitochondrial activity (De Martino et al. 1979) and protein composition (Hecht et al. 1981), may also provide another means for discriminating spermatogenic cells (Petit, et al. 1995). Flow cytometry is widely used for the rapid analysis of various cellular features both in vitro and in vivo. It has emerged as a standard way to analyze solid organs after tissue dissociation in addition to cell lines (Tuomela et al. 2010). Quick screening of testicular phenotypes of transgenic animals, testicular effects of in vivo drug exposure, behavior of cell dynamics in in-vitro cultures of testicular tissue, and in the future for the analysis of human testicular biopsies could also be done with this technique (Rotgers, et al. 2015).

We isolated germ cells from rats 10 days post EDS treatment. The DNA was stained by DNA staining dye VG which binds stoichiometrically to DNA and dyed with dead cell indicator dye PI**.** The use of VG binding specifically and stoichiometrically to N, 2N and 4N cells had a good separation of germ cell subpopulations by DNA content. The N region was easily identified using morphological evolution of the nucleus, which corresponded to the different steps in maturation from the round to the elongated spermatids. The 2N cells were identified as spermatogonia due to their morphology. In the tetraploid region cells were of a similar diameter to 2N cells but the chromatin was uncondensed. Our data suggested that testosterone loss by EDS induces significant germ cell death, a significant decrease in N cells and a decrease in 2N germ cell populations.

Androgens, produced by the testicular LC, act as regulators for initiation and maintenance of spermatogenesis in adult life (McLachlan et al. 2002; Sharpe 1994).

Testosterone binds to the AR, a member of the nuclear hormone receptor transcription factor superfamily, to modulate necessary gene transcription in target cells (Quigley CA et al. 1995). In the adult testis, GC do not express AR, and GCs lacking a functional AR mature normally (Johnston et al. 2001). Recent studies with the Cre/lox system of conditional gene-targeting have stated the cell-specific roles of AR in SC (Chang C et al. 2004; De Gendt K et al 2004), LCs (Xu Q et al 2007), Peritubular myoid cells (PTM; Welsh M et al 2009), and vascular smooth muscle cells (VSM; Welsh M 2010).

Our data with nova red precipitated targeted immune staining showed nAR present in nuclei of SCs, LCs, myoid cells, endothelial cells, and in stripped cytoplasm of elongated spermatids but neither in mitotic nor meiotic germ cells. mRNAs for androgen receptor expression are altered with changes in testosterone level showing that testosterone regulates AR expression is some testicular cells.

The testosterone nuclear receptor (nAR; NR3C4) is coded on the X chromosome that is condensed in spermatogenesis and generally not available for gene expression. This binding of X chromosomal DNA is relaxed only during the mid to late stages of spermiogenesis. Hence, that the nAR is absent in germ cells is well established by nAR antibody studies using a variety of detection approaches. In normal testes germ cells constitute ~80% of the cellular population in rat testis and normal level of testosterone in testicular tissues is about 60-70 ng/ml (10 to 20 times greater than in serum) 10+ times greater than needed for the normal canonical genomic pathways via ARs and the fact that germ cell apoptosis is greatest at 5 to 7 days after LC ablation, we focused our attention

in finding a non-genomic pathway that could bind testosterone and trigger a cascade of germ cell apoptosis and cell loss. Testosterone binds to the ligand binding site of the receptor with the A-ring among the 4 hydrocarbon rings present in testosterone. Progesterone, which is the precursor of testosterone, has a structurally similar A-ring. Due to this similarity in binding patterns, we tried to further investigate the presence of membrane progesterone receptors in testicular germ cells.

The membrane progesterone receptors (mPRs) are members of the progestin and adipoQ receptor (PAQR) family with 7-transmembrane proteins and activate G proteins but do not belong to the G protein-coupled receptor superfamily (Tang YT et al. 2005; Thomas P et al. 2007). Progesterone membrane receptor component 1 (PGMRC1) is a component of nonclassical signaling by progesterone (Losel RM et al. 2008; Rohe HJ et al. 2009; Xu J et al. 2011). Both PGRMC1 and mPRs are reported to have antiapoptotic actions for progestins on granulosa cells (Dressing GE et al. 2010; Peluso JJ et al. 2006). PGRMC1 forms a complex with plasminogen activator inhibitor RNA binding protein (PAIRBP-1) localized on the plasma membranes of spontaneously immortalized granulosa cells (SIGCs) (Peluso JJ et al 2005; Peluso JJ et al. 2006). Higher concentrations of progesterone inhibit the expression of the pro-apoptotic gene BAD and increases the expression of the antiapoptotic BCL2 family member, BCL2A1D, through PGRMC1 in SIGCs (Peluso JJ et al. 2010). In granulosa and breast cancer cells mPRs modulate decreased cell death and anti-apoptotic actions of progestins (Dressing 2010; Dressing et al. 2012). The mPR protein is localized on the plasma membranes of teleost

granulosa cells and on nuclear PR-negative human breast cancer cells (Kelder J et al. 2010). Since our initial studies had provided us with high quality total RNA from testes for all five study groups, we designed primers and tested for the five better known groups of membrane progesterone receptors (mPRs). From our testing, the presence of all five mPRs; mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6), and mPRϵ (PAQR9) were detected in rat testes (Smith, et al. 2008). Out of all five, mPRα (PAQR7), mPRβ (PAQR8), and mPRγ (PAQR5) were expressed more predominantly in testis and gene expressions of all three seems to be dependent upon testosterone concentration. Western blot analysis of mPR alpha, beta and gamma revealed that they were abundant in testicular tissues but vary with change in testosterone concentration among the treatments.

mPRs co-express with nPRs in a wide variety of reproductive tissues, including the myometrium, gonads, gametes, and breast (Dressing, et al. 2007; Karteris, et al.2006; Thomas, 2008; Zhu, Bond & Thomas, 2003). Org 2058 (16α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione), 19-norprogesterone, ethisterone, norethisterone, and norgestrel also display greater binding affinity for nPR than mPRα (Thomas, et al. 2007). With mPRs the greatest affinity found was 18-CH3P4 (Org OE 64-0) with 19-CH2P4 (Org OD 02-0) with second highest binding affinity for mPRα. The mPRα protein is localized on the midpieces of fish and human sperm and regulates progestin upregulation of motility and fertility (Thomas, et al. 2009), suggesting a role in the treatment of male fertility (Kelder, et al. 2010). Progestin binding and signaling through

mPRα has been demonstrated in human myometrial cells (Karteris et al. 2006), in human T lymphocytes during the menstrual cycle (Dosiou et al. 2008), and in rodent gonadotropin releasing hormone (GnRH)–secreting neurons during progestin down-regulation of GnRH secretion (Sleiter, et al. 2009). In our study, after we found 3 mPRs being expressed in testes, we designed experiments to determine testicular cell binding, with testosterone attached to BSA and a fluorescent tag to develop proof of testosterone binding to mPRs.

Our colocalization studies on germ cells isolated from normal rat testis showed presence of mPR-α receptors on the membrane. Progesterone-BSA-FITC and testosterone-BSA-FITC could successfully and distinctly bind to the membrane receptors. BSA coupled with testosterone and progesterone prevented these hormones from perforating through germ cell membrane. When each of these were incubated with cold testosterone, binding of progesterone on mPRs became fuzzy and indistinct. But testosterone binding maintained the distinct rim structure upon binding. Upon EDS treatment, when testosterone was absent in testis, expression of mPRs were reduced and there was little binding of testosterone or progesterone on mPRs which changed its pattern upon cold testosterone treatment. This reduced expression and colocalization reverted to a normal binding pattern upon testosterone replacement post-EDS treatment. Testosterone binding was significantly reduced in EDS treated germ cells. Therefore, it was concluded that binding of testosterone with mPRs is dependent on the concentration of testosterone in testis. Normally, steroidogenesis is primarily in LCs, and testosterone

is the major end product of steroid synthesis. Progesterone is in minimal quantities in rat testis.

A Predicted Model for Alternate Action of Testosterone in Male Germ Cells

As per our previous data in Figure 50, testosterone could successfully bind to mPR alpha. The canonical pathway of testosterone action has been already represented in Figure 18. According to other studies of mPRs on breast cancer cells and oocytes (Pedroza et al. 2020), high concentration of progesterone binds to mPRs instead of diffusing through the plasma membrane to bind to nuclear progesterone receptors.

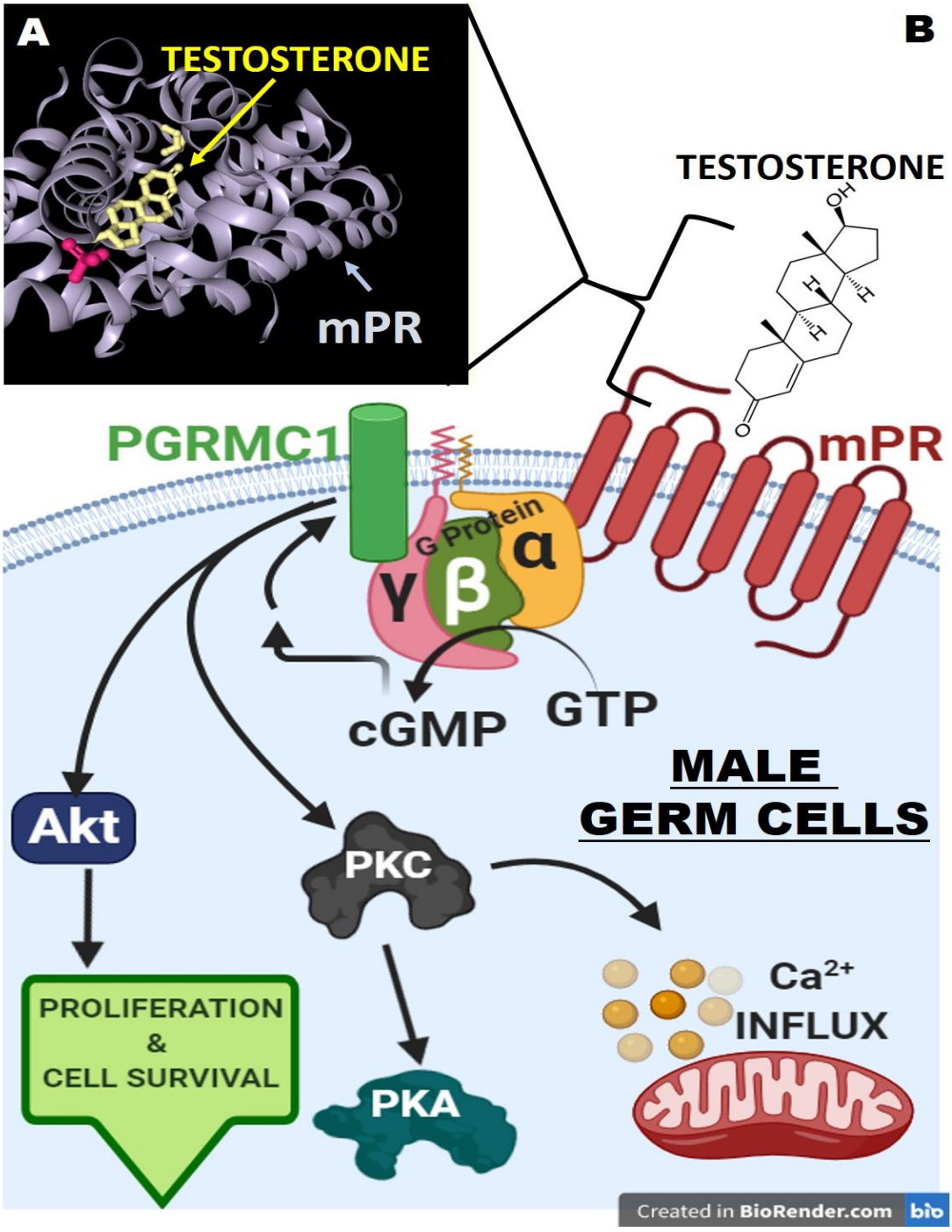
Panel A (see Figure 55) shows protein modelling and testosterone binding to mPR alpha though the A-ring. This model also corroborates with our competitive binding assay/studies (see Figure 50, panel o).

In male germ cells (see Figure 55, panel B), when testosterone binds and activates mPR, the cytosolic part of mPR activates G – protein. This activation leads to conversion of GTP to cGMP. cGMP then activates the cytosolic end of another transmembrane receptor PGRMC1. In breast cancer cell and oocytes, progesterone binds to PGRMC1 to activate it but their protein modelling failed to show any binding capability of PGRMC1 to testosterone. Instead, PGRMC1 could alternatively be activated by cGMP through its cytosolic end. After PGRMC1 is activated, downstream protein AKT (Protein Kinase B) can be activated. AKT is a serine threonine specific protein kinase that plays a crucial role in germ cell proliferation and cell migration. Apart from AKT, PGRMC1 can also activate Protein Kinase C (PKC) involved in phosphorylation of hydroxyl groups of

serine and threonine residues of other metabolic proteins. PKC activates Protein Kinase A (PKA), a cAMP-dependent protein kinase involved in glucose metabolism needed for spermatogenesis and spermiogenesis. These cytoplasmic signaling events could lead to intracellular calcium (Ca^{2+}) influx into mitochondria preventing germ cell death.

Figure 55

A model for alternate action of testosterone in male germ cells.



Note. (A) Protein modelling of testosterone binding with membrane progesterone receptor alpha. Image created by PDB database. (B) Predicted model of testosterone action in male germ cells. It is a non-classical signaling of membrane progesterone receptors (mPRs) and progesterone receptor membrane component 1 (PGRMC1) by activating downstream targets, PKC, PKA, cGMP and AKT leading to Ca^{2+} influx, proliferation, and cell survival. Figure 1 panel B was created using Biorender.

CHAPTER V

CONCLUSION

In summary, we conclude that testosterone is needed to maintain spermatogenesis. LC toxicant EDS triggers germ cell apoptosis depleting T and interfering spermatogenesis. EDS treatment caused significant reduction of *3βHSD2* and *Insl3* transcription in LCs along with testosterone depletion in serum and testes. The resultant testosterone loss increased germ cell apoptosis significantly reducing testicular weight. Flow cytometry data confirmed that haploid germ cells are mostly affected due to loss of testosterone. Germ cells, devoid of testosterone undergo apoptosis by triggering the intrinsic pathway of BCL2 proteins. Testosterone acts mainly through the nuclear androgen receptors present in somatic cells efficiently, as shown in our data (see Figure 48). According to cytogenetic location, the gene for AR is located on the Xq 12, the long arm of the X-chromosome. Though not done in this study, XY genes are thought to be transcriptionally shut down during meiosis thus AR cannot be expressed in germ cells. After meiosis, XY gene expression is reactivated, that is why, ARs were present in the stripped cytoplasm of elongated spermatids. Without the AR in the germ cells, all the testosterone binds to the mPRs present on germ cells. According to gene expression analysis, the expression of mPR-α is reduced with low level testosterone, so may be because of that, we see lesser binding of testosterone with the receptors (see Figure 51 k-t). Lesser number of mPR-α in rat testis without testosterone leads to unsuccessful

binding to exogenously added progesterone-BSA-FITC control (see Figure 51 p-t). This binding phenomenon was rescued to normal in testosterone replaced group EDS + T (see Figure 52). Thus, testosterone binding to mPRs on germ cells serve as an alternate pathway of testosterone action in testis.

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APPENDIX A

List of Abbreviations

LIST OF ABBREVIATIONS

Abbreviation	Full name
ANOVA	Analysis of variance
2N	Diploid Germ Cells
4N	Tetraploid Germ Cells
AKT	Protein kinase B
AMH	Anti-Mullerian Hormone
APC	Anaphase Promoting Factor
AR	Androgen Receptor
ARE	Androgen Response Element
BAD	BCL-2 Associated agonist of cell Death
BAK-1	BCL-2 Antagonist / Killer-1
BAX	BCL-2 Associated X
BCA	bicinchoninic acid
BCL2	B-cell lymphoma 2
BCL-2	B-cell lymphoma-2
BCLW	Bcl-2-Like Protein 2
BCL-XL	B-cell lymphoma extra large

BID	BH3 interacting domain death agonist
BIK	BCL-2 Interacting Killer
BOK	Bcl-2-Related Ovarian Killer
BPA	Bisphenol A
BPM 8B	Bone Morphogenetic Protein 8B
BPS	Bisphenol S
BSA	Bovine serum albumin
BTB	Blood-testis barrier
$C_{(q)}$	Cycle threshold
Ca^{2+}	Calcium
Caspase 9	Apoptosis-Related Cysteine Protease
CD 9	Cluster of Differentiation 9
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CREB	cAMP response element-binding protein
CREM	cAMP response element Modulator
$CrK(SO_4)_2 \cdot 12H_2O$	chromium potassium disulfate dodecahydrate
CSTF 2	Cleavage Stimulatory Factor 2
CYT C	Cytochrome C
DAPI	4',6-diamidino-2-phenylindole

DAZ	Deleted in Azoospermia protein
DDR	Death Domain Receptor
DDT	Dichloro Diphenyl Trichloroethane
DHH	Desert Hedgehog
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DR 3	Death Receptor 3
ED	Endocrine Disruptors
EDS	Ethylene dimethane sulfonate
EDS + T	Testosterone-replaced
EDTA	Ethylenediaminetetraacetic acid
EE	Ethinyl Estradiol
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	Extracellular signal Regulated Kinase
FADD	Fas associated protein with death domain
FAK	Focal Adhesion Kinase
FAS	Fas Cell Surface Death Receptor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

FSH	Follicle stimulating hormone
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Germ Cells
GLUL	Glutathione Synthase
GnRH	Gonadotropin releasing hormone
GnRH	Gonadotropin-releasing hormone
GTP	Guanosine-5'-triphosphate
H&E	Hematoxylin and Eosin
hCG	Human Chorionic Gonadotrophin
HPLC	High Performance Liquid Chromatography
HSD3β2	Beta 3-hydroxysteroid dehydrogenase 2
HSP	Heat Shock Protein
IgG	Immunoglobulin G
IHC	Immunohistochemistry
INSL3	Insulin-like peptide 3
Insl3	Insulin-like Protein 3
Insl3/RLF	Insulin-like Protein 3/relaxin-like factor
ip	Intraperitoneal
ITT	Intratesticular testosterone
LC	Leydig Cells

LDH	Lactate Dehydrogenase
LH	Luteinizing hormone
LH	Leutinizing Hormone
LHR	Luteinizing hormone receptor
LHRH	Leutinizing Hormone Releasing Hormone
LIF	Leukemia Inhibitory Factor
MAP Kinase	Mitogen-activated protein kinase
MCL 1	Induced myeloid leukemia cell differentiation protein
mDF	Modified Davidson's fixative
MLH1	MutL Homolog 1
mPR	Membrane Progesterone Receptor
mRNA	Messenger ribonucleic acid
N	Haploid germ cells
$NaC_2H_3O_2$	sodium acetate
ND	Not detected
NR3C1	Aldosterone Receptor
NR3C2	Glucocorticoid Receptor
NR3C3	Progesterone Receptor
NR3C4	Androgen Receptor
NRE	Negative Regulatory Element
NT	No treatment

ODF 1	Outer dense fiber protein 1
Org 2058	16α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione
P	Progesterone
PAQRs	Progestin Adipo Q Receptor
PAS	Periodic acid–Schiff
PBDE	Poly Brominatd Biphenyl Ethers
PBS	Phosphate buffered saline
PBS	phosphate buffered saline
PCB	Poly Chlorinated Biphenyles
PEN-STREP	Penicillin Streptomycin solution
PGCs	Primordial Germ Cell
PGRMC1	Progesterone Receptor Membrane Component 1
PI	Propidium Iodide
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone Receptor
PRBP	Protamine 1 RNA Binding Protein
PRF	Perforin
PRM 1	Protamine 1
PTM	Peritubular myoid cells
PVDF	polyvinylidene fluoride

qPCR	Quantitative polymerase chain reaction
Raf	Rapidly Accelerated Fibrosarcoma
RBM	RNA Binding Motif
RIA	Radioimmunoassay
ROX	carboxyrhodamine
RT-PCR	Reverse transcriptase polymerase chain reaction
sc	Subcutaneous
SC	Sertoli Cells
sc	Sub-Cutaneously
SCF	Stem Cell Factor
SEM	Standard error of mean
SOD 1	Superoxide dismutase
SP 10	Sperm Protein 10
SPATA 6	Spermatogenesis-associated protein 6
SPRM 1	selective progesterone receptor modulator 1
SRC	Sarcoma Oncogene
SSC	Side scatter
SSO	Sesame seed oil
SSO	Sesame Seed Oil
T	Testosterone-supplemented
T	Testosterone

TDS	Testicular Dysgenesis Syndrome
TdT	Terminal deoxynucleotidyl transferase
TE	Tris-EDTA
TF	Transcriptional Factors
TGF β	Transforming growth factor beta
T_m	Melting temperature
TNF	Tumor necrosis factor
TNF R1	Tumor necrosis factor receptor 1
Tnp1	Transition Protein 1
TP	Testosterone propionate
TPAP	Testis Specific Cytoplasmic Poly (A) Polymerase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end-labeling
VEH	Vehicle treatment
VG	Vybrant Green
VSM	Vascular Smooth Muscle Cells
ZFY 2	Zinc finger Y - chromosomal protein