A SURVEY OF TESTICULAR LESIONS IN STALLIONS

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2008

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2008

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STALLIONS

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ACKNOWLEDGEMENTS

This project was funded by the Bullock Endowment Fund and the NIH Short-Term Training for Health Professional Students Grant to the Oklahoma State University Center for Veterinary Health Sciences. The authors would like to thank Beltex Corporation, 3801 N. Grove, Fort Worth, TX, for providing equine tissues. The authors would also like to thank Dr. Antonio Hinijosa, Dr. Mike Schoonover, Dr. Scotty Howell, Jeff Richards and Kent Kleingarter for their time and assistance in sample collection.

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CHAPTER I

INTRODUCTION

Reproductive management of stallions is extremely challenging, especially when signs of subfertility or infertility develop. Subfertility and infertility contribute largely as an economic loss to the equine industry. Numerous extrinsic and intrinsic factors can disrupt the process of spermatogenesis making management even more difficult.

Testicular degeneration is currently a major concern in the equine industry. Generally speaking, anything that disrupts the process of spermatogenesis results in degeneration. Known insults to the testis such as extreme heat, exogenous steroids, and injury cause degeneration based on the length and severity of the insult. The exact mechanisim of idiopathic testicular degeneration is unknown but it is typically seen in older stallions and involves a defect in the testis itself resulting in degeneration.

An element of surprise is often involved with stallions that develop testicular degeneration. Most owners don't know it's happened until mares fail to become pregnant. Many times at this point, the damage is irreversible and fertility will continue to decline over time.

The study described herein is important in defining and recording histopathologic changes found in the testes of a general population of stallions. These results combined with future studies will attempt to define testicular changes that take place with the aging

process. Improvements to breeding soundness examinations could result in earlier detection of testicular degeneration and although it can't be stopped, proper planning could prepare stallion owners for what's to come.

CHAPTER II

REVIEW OF LITERATURE

To properly evaluate and manage a stallion suspected of having reproductive problems, a thorough understanding of reproductive anatomy, physiology, endocrinology and histology is fundamental. Part I of the following literature review will focus on these elements in the stallion thus providing a foundation for Part II which will focus on testicular degeneration in the stallion.

PART I

Functional Anatomy & Histology of the Adult Stallion

The reproductive tract of the male is supported partially within the pelvic cavity by the genital fold and externally by the scrotum and prepuce. The male reproductive organs consist of the following: two testes, each suspended by a spermatic cord and external cremaster muscle; two epididymides; two ductus deferens each with an ampullae; paired vesicular glands; a prostate gland; paired bulbourethral glands; the penis and associated urethralis; ischiocavernosus, bulbospongiosus, and retractor penis muscles. The vesicular glands, prostate gland and bulbourethral glands are often collectively called the accessory sex glands.

Scrotum

The scrotum in a normal stallion should be slightly pendulous, spherical, and symmetric with testes location varying between stallions. The scrotum should be thin and flexible, sliding easily over the testes and epididymides inside.

The scrotum functions mainly by protecting the testes and assisting in thermoregulation. The scrotum of a stallion is located high within the inguinal area and consists of four main layers including 1) skin, 2) tunica dartos, 3) scrotal fascia, and 4) parietal vaginal tunic [1-4]. Scrotal skin is thin, hairless, and slightly oily and contains numerous sweat glands and sebaceous glands [1,5]. The tunica dartos layer lines the scrotum and is composed of smooth muscle fibers and connective tissue. It is capable of constantly adjusting to temperature changes and is able to maintain continuous contractions for long periods of time.

The scrotal fascia lies between the tunica dartos and the parietal vaginal tunic. This layer consists of a movable connective tissue layer that allows vertical and horizontal movement of the testes within the scrotum [4]. The inner most layer of the scrotum is the parietal vaginal tunic or the common vaginal tunic which forms through an evagination of the parietal peritoneum through the inguinal rings when the testes descend. The vaginal cavity, a space between the parietal and visceral layers of the vaginal tunic, contains a fluid that allows movement of the testes within the scrotum. Adhesion formation between these two layers is common in older stallions due to normal movement and mild trauma over time. These adhesions can ultimately inhibit mobility and prevent proper thermoregulation.

Testis

The testis functions mainly in the production of both spermatozoa and testosterone. In a normal stallion, the testes are oval shaped, relatively equal in size and firm on palpation. In stallions, the testes lay horizontally in the scrotum with the tail of the epididymis directed caudally. The ligament of the tail of the epididymis is a remnant of the fetal ligament called the gubernaculum, which is thought to be involved in descent of the testes into the scrotum. The ligament attaches the tail of the epididymis to the caudal pole of the testis and is predominantly large in newborn colts, often mistaken for a testis in the scrotum [4].

The tunica albuginea is a tough layer of collagenous tissue and smooth muscle that encloses each testis within the scrotum and is fused externally to the visceral layer of the vaginal tunic. The testicular parenchyma is separated into lobules by supportive trabeculae from the tunica albuginea [2,4,5]. The smooth muscle layer of the tunica albuginea functions in intratesticular sperm transport and testicular tone [6]. At the cranial pole of the testis lies the mediastinum testis, a partial separation inside the testis, consisting of fibrous tissue that is continuous with the tunica albuginea. The stallion mediastinum is located axially in the testis when compared to other species [1,2,4]. Excurrent ducts exit the testis through the mediastinum, cross the tunica albuginea and enter the head of the epididymis [2,5].

The testis parenchyma of a young stallion is much more pale when compared to the parenchyma of older stallions [7]. The parenchyma contains seminiferous tubules and interstitial tissue. Seminiferous epithelium lines the inside of the seminiferous tubules and contains germinal cells and Sertoli cells. The lamina propria surrounds the

seminiferous tubules and consists of fibroblasts, myoid cells and laminin. The myoid cells move spermatozoa and fluid through the tubules by producing rhythmic contractions. Sertoli cells form junctional complexes and also assist germinal cells in differentiation. The junctional complexes form a blood-testis barrier that separates the developing cells from the host immune system.

The interstitial tissue contains blood vessels, lymphatics, nerves, connective tissue and large numbers of Leydig cells which produce testosterone among other steroid hormones [7]. As a stallion reaches sexual maturity, Leydig cells are capable of producing larger amounts of testosterone.

Seminiferous tubules are arc shaped and consist of three zones 1) convoluted tubule zone, 2) transitional tubule zone and 3) straight tubule zone. The convoluted zone is highly coiled, making up a major portion of the seminiferous tubule. This zone is lined by seminiferous epithelium, which contains Sertoli cells and germinal cells capable of spermatozoa production. The seminiferous epithelium of an adult stallion is made up of 15-20% Sertoli cells that are able to multiply as breeding season approaches [7-10]. The number of Sertoli cells within the testis determine how many spermatozoa are produced [11].

The transitional zone lies between the convoluted and straight tubule portions of the seminiferous tubule. The straight tubules unite in the cranial 2/3 of the testis at the rete testis [11,12]. These tubules then join with the efferent ducts that lead to the epididymal duct.

Seasonal breeders like the stallion experience hormonal and testicular changes throughout the year but unlike some seasonal breeders, stallions produce spermatozoa year round. The peak breeding season for stallions is from May-July with regression evident in the nonbreeding season from September-February. During the nonbreeding season, testes are 25% lighter, contain 35% less Leydig cells which produce less testosterone, contain 31% less Sertoli cells and produce 40-50% less spermatozoa [13-15].

Torsion & Rotation of the Testis

Normal stallions can commonly have rotation in one or both testes, up to 180 degrees. A study showed that 3-4% of light horse stallions presented for a breeding soundness examination had some rotation present in addition to evidence of decreased sperm motility and extragonadal sperm reserves [16]. Rotation is usually prevented by the loose connective tissue inside the scrotum with the condition being more prevalent in certain breeds [6,16,17]. In a study evaluating Welsh ponies, rotation was evident in 39% of the stallions examined. This high incidence is thought to be related to a specific family lineage [18]. The incidence of testis rotation in Paso Fino stallions is about 15%. The testis rotation in stallions with this condition is typically permanent and no outward signs of distress are present [19].

Testis rotation should not be confused with true testicular or spermatic cord torsion which is often serious and life threatening. The incidence of testicular torsion in stallions is unknown; however, in humans, one in every four thousand males younger than twenty-five years old is diagnosed with this condition annually [20]. Testicular torsions in stallions often result in signs of colic with the affected testis presenting painful and swollen. One study evaluated a stallion with a three year history of left sided scrotal pain

and swelling. After removal of the testis for testicular torsion, the stallion returned to service with an 82% conception rate the next season [21].

Spermatic Cord & Vascular Supply to the Testis

The spermatic cord is enclosed in the parietal layer of the vaginal tunic and travels distally from the internal inguinal ring. Each cord contains a deferent duct, testicular veins, testicular artery, nerves, and lymphatic vessels. The cremaster muscle is not part of the spermatic cord but lies lateral to it [2,4].

The testicular artery is a branch of the abdominal aorta, supplying blood to the testis and epididymis. It descends through the inguinal ring and runs along the cranial border of the spermatic cord in a winding manner, dividing into numerous branches close to the testis. The small branches pass through the tunica albuginea and enter the testis parenchyma through the trabeculae and septa [1,3,4]. The testicular artery intricately winds around a complex system of veins leaving the testis to form the pampiniform plexus. The pampiniform plexus ultimately joins the caudal vena cava [1,3,4].

An abnormal distension of the veins of the pampiniform plexus is referred to as a varicocele. This condition is relatively rare in stallions [5] but common in male humans with the condition present in 12% of the normal male population and in approximately 25% of men with infertility [22]. In stallions with the condition, the vessels are distended on palpation, usually not painful, and typically only involve one side of the spermatic cord. The condition is thought to alter spermatogenesis by changes in thermoregulation [6] but stallions with the condition can have normal semen parameters [5]. Although

surgery is typically not performed in the stallion, there is much debate in human medicine whether men with infertility would benefit from surgical correction of the condition [22].

Excurrent Duct System

Each of the rete tubules joins an efferent duct after passing through the tunica albuginea, continuing on to the epididymal duct and finally into the deferent duct. The deferent duct ends at the colliculus seminalis, a rounded prominence situated on the dorsomedial wall of the urethra, caudal to the urethral opening from the bladder [4]. It is at this prominence that the ducts of the accessory sex glands empty into the urethra.

Epididymis

Each testis has a highly convoluted epididymis, which is approximately 70 meters in length [2,4,5]. The three grossly distinct regions of the epididymis are the head, body, and tail. The head (or caput) of the epididymis is a flattened structure that is closely attached to the testis. It curves around the testis and lies lateral to the spermatic cord. This region of the epididymis can be difficulat to palpate die to its flattened nature and relation to the cremaster muscle [5,23]. The body (or corpus) is cylindrical in shape and is loosely attached to the dorsal surface of the testis. The tail (or cauda) is a large, spherical, prominent structure attached to the caudal pole of the testis by the ligament of the tail of the epididymis.

The proximal head of the epididymis contains the distal ends of numerous efferent ducts leading out of the tubules and into the rete testis [12]. The efferent ducts fuse into the epididymal duct within the head of the epididymis. The epididymal duct is approximately 45 meters long, is folded in pleats and courses through the head, body and

tail of the epididymis becoming continuous with the deferent duct. Multiple regions within the stallion epididymis can be identified based on cellular structure with each region having a different function [24].

Functionally the epididymis has three sections [25-28]. Within the proximal head of the epididymis, the epithelia of the efferent ducts function in resorption of fluids. Spermatozoal maturation takes place in the distal part of the head of the epididymis and the body where specific secretions are released. The tail of the epididymis and the proximal aspect of the deferent duct both function as storage facilities for fertile spermatozoa.

Deferent Ducts

The deferent duct, a continuation of the epididymal duct, attaches to the tail of the epididymis. It courses along the medial aspect of the testis and ascends via the spermatic cord through the vaginal ring into the pelvis. Each deferent duct widens into an ampullary gland, eventually concluding at the colliculus seminalis of the pelvic urethra. The proximal deferent duct has a thick wall of smooth muscle that can be easily palpated through the scrotum.

Accessory Sex Glands

The accessory sex glands collectively include the ampulla, vesicular glands, prostate gland and the bulbourethral glands.

Ampullae

The paired ampullae are the distended distal portions of the deferent ducts, measuring 1-2 cm in diameter and 10-25 cm in length [5,23]. They can be palpated on midline of the

pelvic floor over the neck of the bladder. They lie dorsal to the pelvic urethra but pass beneath the prostate gland as they join caudally. They continue through the wall of the urethra at their distal ends, opening into the colliculus seminalis alongside the excretory ducts of the vesicular glands. There is much debate regarding whether the ampullae are simply a storage depot for sperm or an actual accessory sex gland [29]. Histologically, the tissue is composed of numerous branched tubular glands within thickened walls [4,6,30].

Vesicular Glands

The paired vesicular glands are pyriform sacs with thin walls that lie lateral and parallel to the ampullae within the genital fold [31]. The glands range in size from 12-20 cm long and 5 cm in diameter [1,5] with the ducts opening lateral to the ducts of the ampullae at the colliculus seminalis. The vesicular glands produce the gel fraction of the ejaculate [5,32]. Season can influence the volume of gel produced often with the highest fraction of gel being produced during the physiologic breeding season [15]. The volume of the gel fraction can also vary significantly between stallions.

Seminal vesiculitis is a unilateral or bilateral disease that rarely occurs in stallions but is significant due to its persistent nature and interference with fertility [33]. Typically, clinical signs are not evident, although some stallions exhibit pain on ejaculation or a reluctance to breed [5]. In a case report of a stallion with seminal vesiculitis, presenting clinical signs were those of colic [34]. Some stallions have enlarged vesicular glands that are firm and painful on rectal palpation. Pozor and McDonnell utilized ultrasound to analyze the echogenic character of vesicular fluid and found a significant variation within and between stallions that were all known to have semen free of inflammatory cells. This

led them to conclude that a change in the echogenicity of vesicular fluid doesn't necessarily indicate inflammation [31]. Vesicular glands of stallions can vary considerably in size and appearance both across and within stallions [31]. Diagnosing seminal vesiculitis is best done with a combination of rectal palpation, observation of large numbers of neutrophils in the semen, bacterial culture of the semen, and endoscopy of the urethra and seminal vesicles [5,35]. Culture swabs should be taken from the preputial cavity and also from the penis before it is washed to determine the resident bacterial flora in these areas. The pre- and post-ejaculate swabs are taken from the distal urethra in addition to semen cultures. Proper techniques will ensure that the artificial vagina is not the source of contamination [5]. Endoscopy and direct cultures of the seminal vesicles will result in a more significant diagnosis [35,36].

Seminal vesiculitis is often difficult to treat and the prognosis is guarded. Affected stallions can be treated with systemic antibiotics and their semen should be extended with a product containing antibiotics. Several reports have promoted endoscope-aided direct lavage followed by the placement of antibiotics into the vesicular gland lumen [35,36].

Prostate Gland

The prostate is firm, has two lateral lobes, and a central isthmus. It extends along the caudolateral border of each vesicular gland and is not always palpable per rectum. The lobes measure between 5-9 cm in length, 2-6 cm wide, and 1-2 cm in thickness [2,5]. The prostate ducts enter the lumen of the urethra lateral to the colliculus seminalis and contribute to the sperm-rich fraction of the ejaculate [5,32].

Bulbourethral Glands

The bulbourethral glands lie caudal to the prostate and are attached to the dorsal surface of the pelvic urethra. They are difficult to palpate per rectum because the urethralis and bulboglandularis muscles cover them [1,4], but ultrasonographically, they are easily evaluated [31,37]. Multiple ducts from the bulbourethral glands enter the medial side of the urethra distal to the prostatic ducts. The bulbourethral gland secretions make up a majority the first fraction of the ejaculate [32], functioning to cleanse the urethra prior to ejaculation [15].

Urethra

The urethra extends from the bladder to the free end of the penis and functions as an outflow tract for both urine and semen. The urethralis muscle overlays the pelvic portion of the urethra and contracts powerfully during ejaculation. The urethra is typically narrow except at the location of the colliculus seminalis where it widens to allow deposition of the accessory sex gland fluids during ejaculation. The urethra ends in a free extension called the urethral process. The penile urethra is surrounded by the corpus spongiosum penis.

Penis & Prepuce

The stallion penis is musculocavernous in nature and is composed of a root, a body, and a glans penis [1,2,4,15]. The ischiocavernosus muscles and suspensory ligaments of the penis support the root of the penis. At the ischial arch, the penile root arises in the form of two crura which combine distally to form the single and dorsal corpus cavernosum penis which is enclosed by a thick tunica albuginea. The corpus cavernosum, corpus spongiosum, and corpus spongiosum glandis are the cavernous spaces making up the

erectile tissue of the penis. An erection occurs when blood from branches of the internal and external pudendal arteries engorge the spaces [1,3]. These cavernous spaces are also continuous with the veins responsible for drainage. At the pelvis, the corpus spongiosum begins as the bulb of the penis and distally encircles the penile urethra inside a groove on the ventral side of the penis. It continues distally over the free end of the penis to form the glans penis. The corpus spongiosum glandis forms the distinct bell shape of the stallion's penis seen following coitus. At the center of the glans penis the urethral process is noticeably visible and is surrounded by an invagination known as the fossa glandis.

The bulbospongiosus muscle lies ventral to the urethra and runs the entire length of the penis. This muscle is a direct continuation of the urethralis muscle and assists in moving the penile urethral contents (semen and urine) distally with smooth rhythmic contractions. The paired retractor penis muscles are smooth muscles that course ventrally along the penis and join at the glans penis. They function by returning the penis to the sheath following erection.

The prepuce is formed by a double fold of skin and functions to surround and protect the nonerect penis. The preputial skin is essentially hairless and has an abundant amount of sebaceous glands and sweat glands [2,4,5]. The sheath (external part of the prepuce) begins at the scrotum and has an evident raphe that is continuous with the scrotal raphe. The preputial orifice is formed by the sheath reflecting dorsocaudad to the abdominal wall [4]. The internal layer of the prepuce extends caudad from the orifice to line the internal side of the sheath, and then reflects craniad toward the orifice again before reflecting caudad to form the internal preputial fold and preputial ring. This additional

internal fold allows the distinct lengthening (~50%) of the stallion's penis during erection. The preputial orifice is located at the base of the penis just in front of the scrotum during erection with the preputial ring evident near the midshaft of the penis. Also during erection, the internal layer of the internal preputial fold is distal to the preputial ring.

Reproductive Physiology & Endocrinology of the Stallion

The production of spermatozoa capable of fertilization is essential to reproduction. Spermatogenesis is an extremely delicate process that can be easily disrupted. A thorough understanding of reproductive function is critical so that actions interfering with normal testicular function can be avoided and causes of altered spermatozoa production can be identified. Endocrine function is an important aspect of stallion reproduction that can be easily disturbed with the administration of exogenous hormones.

Thermoregulation of the Testes

Most mammals accomplish testicular thermoregulation by scrotal sweat glands, scrotal muscle relaxation and the arteriovenous countercurrent heat exchange mechanism at the pampiniform plexus [38]. Normal spermatogenesis occurs at a temperature several degrees below core body temperature making proper thermoregulation a necessity [6,15,38].

Sweat glands in the scrotum are innervated by sympathetic nerves that assist in proper testicular temperature. If the body or scrotal temperatures increase, the hypothalamus detects the change and transmits nerve impulses to the sweat glands. Sweating allows the scrotum and thus the testes to be cooled by evaporation. The tunica dartos and the

external cremaster muscle also play a role in thermoregulation by determining testis position. This is relevant during times of hot and cold temperature extremes. During cold winter months, the tunica dartos contracts allowing the testes to be held closer to the body for increased warmth and in warmer months the tunica dartos relaxes making the scrotum more pendulous and thus increasing the surface area of the scrotum for evaporative cooling. These mechanisms in combination with an increase in scrotal perspiration allows for a greater rate of evaporation and more efficient cooling of the testes. The external cremaster muscle is also able to raise the testis for short time intervals. Androgens (testosterone) are necessary for the tunica dartos to function, thus castrated animals lose this ability. The pampiniform plexus also aids in thermoregulation by serving as a countercurrent heat exchanger. The cooler venous blood leaving the testis enters the pampiniform plexus where it intricately winds around the testicular artery, and functions by cooling the arterial blood as it enters the testis preventing heat shock and damage [5,15]. When comparing the scrotal skin temperature to testis temperature, the skin is 33°C while the testes are 30.5°C to 32.5°C [15].

Increases in scrotal and testicular temperature are known to affect spermatogenesis and have been experimentally induced and investigated in bulls [39-43], rams [44-48], boars [49-51] and rabbits [52-54]. Love and Kenney conducted a study to determine the effects of scrotal heat stress on four pony stallions. Semen was collected and evaluated prior to the placement of an airtight, wool apparatus over the scrotum and testes. The apparatus was kept in place for 48 hours and then removed. Semen was collected and evaluated for 64 days following removal to ensure a complete spermatogenic cycle had taken place. It

was found that sperm within the epididymis was not altered whereas the primary spermatocytes appeared to be the most susceptible [55].

Spermatogenesis

Spermatogenesis is a long process by which spermatogonia divide by mitosis to produce a population of spermatogonia that will maintain the cell line in addition to producing differentiated spermatogonia. These differentiated spermatogonia then divide by mitosis to produce primary spermatocytes. The spermatocytes undergo meiosis to produce spermatids which finally differentiate into spermatozoa.

Spermatogenesis occurs within the seminiferous epithelium of the convoluted seminiferous tubules. The seminiferous epithelium of a mature stallion consists of various cells including, somatic cells called Sertoli cells, and different types of germinal cells including spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. Within a normal seminiferous tubule, there are four to five generations of developing germinal cells that are arranged in distinct cellular stages [56-59]. Each of these generations is 12.2 days more highly developed toward forming spermatozoa [60]. The length of spermatogenesis is not influenced by season and is approximately 57 days in stallions [15,57,60,61]. If any event interferes with spermatogenesis, at least 2 months may be required before normal function is returned.

The process of spermatogenesis (57 days) consists of three phases including 1) spermatocytogenesis (19.4 days) is characterized by stem cell spermatogonia dividing by mitosis to produce other stem cells that will continue the lineage throughout the adult life of the male and also dividing cyclically to produce committed spermatogonia, 2) meiosis

(19.4 days) is characterized by the exchange of genetic material between homologous chromosomes of primary spermatocytes and is followed by a reduction division producing haploid spermatids, 3) spermiogenesis (18.6 days) is characterized by differentiation of spermatids with spherical nuclei into spermatozoa which are released from the luminal free surface.

Sertoli Cells

The Sertoli cells can be found on the lamina propria of the seminiferous tubule with their widespread cytoplasm extending around the germinal cells into the tubular lumen. Sertoli cells play a key role in spermatogenesis but their exact function is still unknown. The number of Sertoli cells a testis contains relates to the amount of spermatozoa that can be produced [10,11,57,62-65]. Sertoli cells have numerous functions that include [65], blood-testis barrier formation; nutritional and structural support of germinal cells; movement of developing germinal cells within the seminiferous epithelium; release of mature spermatids by spermiation; phagocytosis of degenerating germinal cells and residual bodies of cytoplasm left behind as mature spermatids undergo spermiation; secretion of fluids and proteins to bathe the developing germinal cells and convey spermatozoa through the seminiferous tubules to the rete testis; cell-to-cell communication with developing germinal cells, the underlying myoid cell layer (part of lamina propria), and Leydig cells.

The organization of germinal cell differentiation and separation of the germinal cells from the host stallion's immune system are both significant functions of Sertoli cells. The blood-testis barrier is formed by tight junctional complexes that are located between adjacent Sertoli cells. The junctional complexes divide the seminiferous epithelium into

two functional compartments: basal (peripheral) and adluminal (inner). Spermatocytogenesis takes place within the basal compartment, where spermatogonia and primary spermatocytes are found. In the early phase of meiosis, primary spermatocytes migrate through the blood-testis barrier, into the adluminal compartment where meiosis continues and spermiogenesis occurs. Integrity of the blood-testis barrier is maintained by forming new junctional complexes below the primary spermatocytes before dissolution of junctional complexes above the cells.

The spermatocytes and spermatids are protected from the host immune system by the blood-testis barrier. The differentiated spermatocytes and spermatids are considered foreign to the body because the developing immune system is not exposed to them and it would react to antigens expressed on their surface. This barrier controls fluids and molecules entering so spermatocytes and spermatids are protected. Damage to this barrier is rare but if it occurs, orchitis results. Spermatozoa outside of the seminiferous tubule are recognized by the immune system as foreign. The rete testis and efferent ducts are common sites for orchitis, which often begins with a granulomatous reaction [66]. Human males can form antispermatozoan antibodies when there is a major disruption in the blood-testis barrier. Infections, trauma and neoplasia that damage the testis can result in antibody formation. Stallions experiencing trauma to the testis with resulting low sperm cell viability have been found to have antispermatozoan antibodies in their seminal fluid or serum [66].

It is common to find a multifocal, mild, subacute intertubular inflammation in bulls and stallions with no gross lesions evident [66]. In stallions, mild interstitial orchitis is common with interstitial lymphocytic foci, often perivascular, occurring in areas of

tubule degeneration and vasculitis [66,67]. Similar lesions have been found to be part of a generalized vascular association in equine viral arteritis [67]. A focal interstitial orchitis can commonly be found in human testes removed at autopsy and also in testes from prostate cancer patients; however, the cause of inflammation is unknown. Atrophy of the germinal epithelium is often associated with severe interstitial orchitis and if bilateral may result in infertility [68].

Intratubular orchitis most likely arises from an ascending infection of the urethra, urinary bladder, ductus deferens and epididymis [66,67]. This inflammation typically begins in the seminiferous tubules but spreads to the interstitium resulting in granuloma formation when spermatozoa breach the tubule border. The seminiferous tubule outline is often preserved in the affected area but the seminiferous epithelium is destroyed and replaced by abundant macrophages and multinucleated giant cells that surround neutrophils and debris [67].

Sertoli cells are in close contact and control the immediate environment around all germinal cells with the exception of spermatogonia [69-72]. Sertoli cells contain several unique proteins involved in changing the formation of spermatozoa and also play an important role in producing proteins to carry iron, copper and vitamin A to developing germ cells. It is hypothesized that a disruption in spermatogenesis affects Sertoli cells rather than the germinal cells directly. The number of Sertoli cells per testis and the maximum number of germinal cells per Sertoli cell is predetermined within each species [73,74] thus the number of Sertoli cells per testis is a highly heritabile trait [11,62]. Johnson et al. found results consistent with the hypothesis that the number of Sertoli cells is important in determining testicular size and daily spermatozoal production and that the

relationship of daily spermatozoal production to the number of Sertoli cells or to parenchymal weight has been established as early as the level of primitive spermatogonia [75]. Spermatozoal production could be adversely affected by any treatment that alters the function or number of Sertoli cells before puberty

Germinal cells are supported by Sertoli cells through close contact, interdigitation of plasma membranes, and cellular specializations allowing communication. In the process of spermiogenesis, the Sertoli cells move spherical spermatids by microtubular and microfibrillar movements first toward the basal membrane and then back towards the luminal side. Sertoli cells are partially stimulating spermatids to change shape at this time too.

The excess cytoplasm, known as a residual body, is phagocytized by surrounding Sertoli cells. The spermatozoa are now mature but are not capable of fertilization. They are released by the Sertoli cells in the lumen of the seminiferous tubule in a process called spermiation. Any germinal cells that die during spermatogenesis are also phagocytized by the Sertoli cells.

Sertoli cells produce various secretions that surround and nourish the germinal cells in addition to secretions that make up a component of the luminal fluid of the seminiferous tubule. Sertoli cells produce unique secretions not found anywhere else in the body in addition to secretions similar to those from the epididymis and other accessory sex organs. Some secretions such as lactate serve as an energy source for developing germinal cells, some help in the regulation of epididymal function and some serve as transport molecules to move essential metals, vitamins, or hormones from Sertoli cells to

developing germinal cells. Sertoli cells secrete the protein hormone inhibin from two locations 1) from the luminal surface through the epididymis in the rete testis fluid of the seminiferous tubule and 2) through the basal surface through the interstitial fluid of the testis where it ultimately enters by the lymphatic and venous drainage.

Follicle stimulating hormone (FSH) and testosterone are responsible for directing the function of Sertoli cells. Sertoli cells have also been shown to communicate with both Leydig cells and germinal cells. Leydig cells produce a substance that has an effect on both Sertoli cells and Leydig cells, terminating mitosis of indifferent supporting cells before puberty [76]. Insulin-like growth factor (IGF), epidermal growth factor (EGF), and transforming growth factor-β (TGF-β) also play important roles in regulating testis function. Sertoli cells produce mitogenic polypeptides that stimulate or coordinate mitosis and meiosis of germinal cells [77,78].

Cycle of the Seminiferous Epithelium

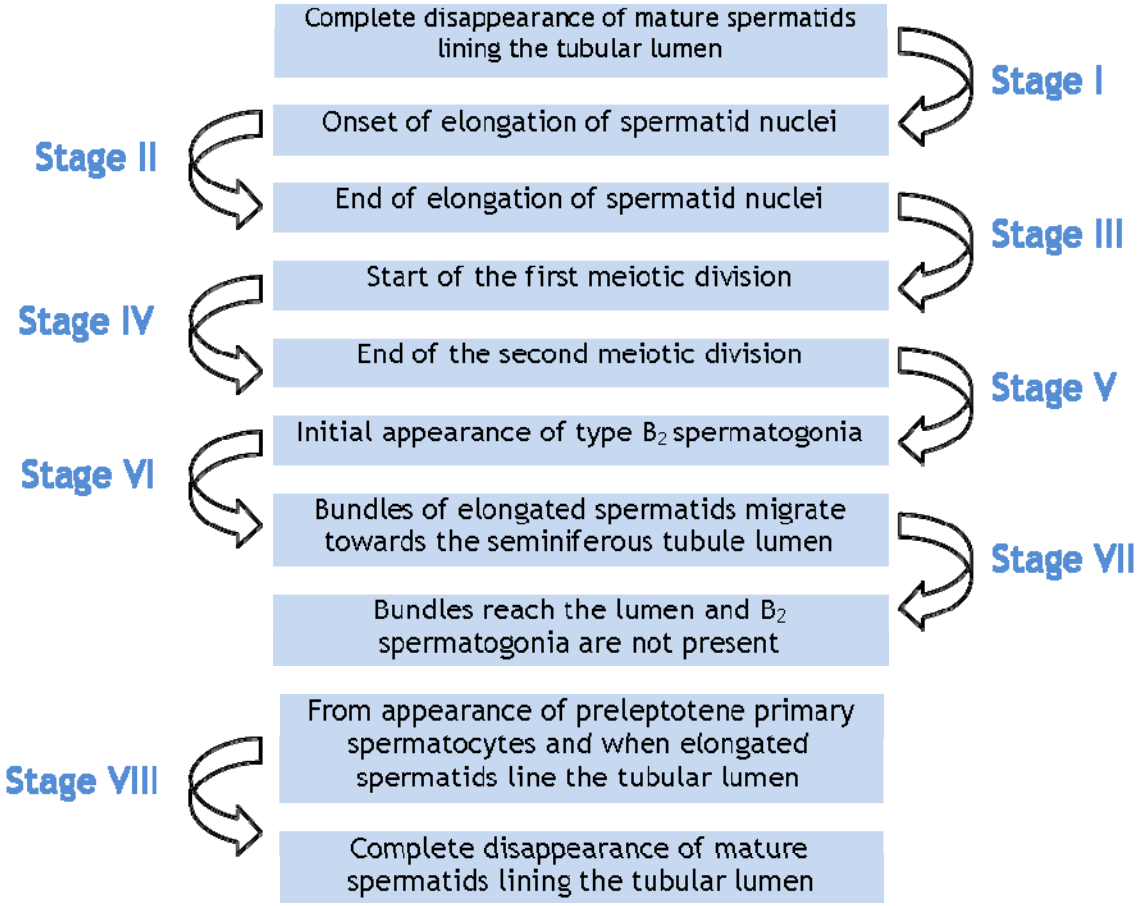
Studies evaluating histologic sections of stallion testes found differences between adjacent cross sections in the seminiferous tubules. Eight cellular stages have been defined based on four to five specific types of germinal cell groupings [56-59]. The exact number of cellular stages depends on the criteria used for identification of each grouping of germinal cells [57,69,72,79-84]. Four or five types of germinal cells are associated with a specific layer within each cellular stage with each layer representing one generation of germinal cells that is 12.2 days more developed than the layer below it. Young generations are located along the outside edge of the seminiferous tubule at the lamina propria while the older generations are closer to the lumen of the seminiferous

tubules. All normal testes have germinal cells that can be found in these specific cellular stages.

If an area within a stallion seminiferous tubule were watched over time, there would be a sequential development of the germinal cells through all eight of the cellular stage's [15,56-59]. One cycle of the seminiferous epithelium progresses through each cellular stage. This process then occurs over and over again in an expected manner. The duration of the cycle of the seminiferous epithelium is the time it takes for one complete series of cellular stages to occur at one point within the tubule.

In a study evaluating spermatogenesis in twelve stallions, Swierstra et al. identified all eight stages of the seminiferous epithelium based on the structure of the germinal cells and their position within the epithelium [59]. See Figure 1 below.

Figure 1 Descriptions of the Eight Cellular Stages



The relative rate of occurrence of the eight stages is expressed as a percentage of the total number of tubular cross sections evaluated for each testis. Two generations of primary spermatocytes are found in stages I through III, and thus about 35% of cross sections through seminiferous tubules should contain two generations of primary spermatocytes in a normal testis. Also, two generations of spermatids are found in stages V through VIII so that about 50% of cross sections in a normal testis should contain two generations of spermatids, and 16% should have spermatids lining the tubular lumen (stage VIII).

Cross sections through a seminiferous tubule often contain a single cellular stage wheras others contain two or three cellular stages [56,58]. This most likely represents locations where different cellular associations adjoin [60,69]. Swierstra et al. measured the duration of a cycle of the seminiferous epithelium in six stallions using a radioisotope called [^{3}H]thymidine [58,59]. The radioisotope was injected into both testicular arteries of the stallions. Germinal cells developing and synthesizing DNA at the time of injection incorporated the [^{3}H]thymidine and were able to be identified based on radioactivity. The six stallions were castrated 4.5 h to 35 days following the injection. A photographic emulsion was used to coat the histologic testicular sections and identify the radioactive germinal cells. After the film was taken and developed, black "grains" were evident indicating radioactive germinal cells.

Samples collected 4.5 h after injection of [^{3}H]thymidine showed that young (leptotene) primary spermatocytes in stage I were the most mature radioactive germinal cells. Stallions castrated at >4.5 h after injection of the radioisotope had progressively more advanced germinal cells labeled. Swierstra et al. concluded after analyzing the data that the duration of one cycle of seminiferous epithelium was 12.2 days [58,59]. Therefore at any given point within a seminiferous tubule, the same cellular stage will be repeated every 12.2 days.

Duration of Spermatogenesis

Within a seminiferous tubule, A_1 spermatogonia periodically produce committed $A_{1\text{-}2}$ spermatogonia. This occurs at an interval equal to the duration of one cycle of the seminiferous epithelium [15,57,60,69,72,81,84]. Also at some point in the cycle, groups of mature spermatids, originating from spermatogonia committed to differentiate

approximately 4.7 cycles earlier are released from the seminiferous epithelium. Thus, because of the division of A_1 spermatogonia every 12.2 days [58], a new group of germinal cells begins to develop every 12.2 days, with all of the cells in this group developing together. The process of spermatogenesis requires about 57 days in the stallion, which calculates to approximately 4.7 cycles of the seminiferous epithelium (12.2 days). Since A_1 spermatogonia are developing at different times at different sites within the testis, hundreds of spermatogonia committed to form spermatozoa are produced every second. This process makes it possible for spermatozoa to be released continuously from the seminiferous tubules.

Daily Spermatozoal Output

Breed, age, season and reproductive status all affect the testicular size in a stallion [15,85-87]. Testis size is an accurate indicator of spermatozoal production with small testes yielding a lower sperm output than larger ones. A correlation exists between testis parenchymal weight and daily spermatozoal production making it a useful predictor of a stallion's breeding potential [16,87-90]. Season significantly impacts daily spermatozoal production in 6-20 years old stallions with an average decline of 50% during the nonbreeding season (6.40 vs. 3.19 billion spermatozoa per day). Stallions slaughtered in September-February have the lowest daily spermatozoal production compared to maximum daily spermatozoal production in May and June [10,57].

The number of spermatozoa produced per gram of testicular parenchyma defines efficiency of spermatozoal production. Efficiency during the breeding season is similar among normal stallions [10,57,63,64,91-93] and on average is about 19 million spermatozoa per day per gram of testis, declining to about 15 million spermatozoa per

day per gram of testis in the nonbreeding season [7,10,11,57,63,91-94]. Stallions that are 13-20 years old experience a 49% decrease in daily spermatozoal production but only a 20% decrease in testis weight [63].

Testicular volume is estimated by testicular measurements and correlates with sperm production. Estimates of this value must be used since parenchymal weight cannot be established in a live stallion without castration first. Testis size can be estimated using calipers or ultrasound [5,15,90,95]. A study by Bailey et al. compared caliper and ultrasound measurements of ten bulls to determine which method was the most accurate. It was found that caliper length measurements were more reliable than ultrasound derived lengths. Width measurements were comparable between the two methods [96]. Caliper error is also possible and can occur with operator technique, caliper sensitivity, and testis location within the scrotum [87,90,94]. The average of several measurements helps increase repeatability and accuracy.

Total scrotal width measurements in the stallion correlate well with testis parenchymal weight and daily spermatozoal production [87,94]. The use of a single linear measurement to determine a three-dimensional structure is of questionable use [90]. One study suggested that testicular volume, rather than dimensions, more accurately predicts daily spermatozoal output since the testis is ellipsoid [97]. The following formula converts length, width and height measurements into testicular volume [97]:

$$\text{Testis Volume} = \frac{4\pi}{3} \times \frac{\text{Length (cm)}}{2} \times \frac{\text{Width (cm)}}{2} \times \frac{\text{Height (cm)}}{2}$$

Love also recommends using this volume to predict the expected daily sperm output of the stallion, using the following formula [97]:

$$\text{Predicted Daily Sperm Output} = 0.024 \times (\text{Left Volume} + \text{Right Volume}) - 0.76$$

In males of most species, daily spermatozoal production has a moderate to high correlation with daily spermatozoal output [98]. The predicted daily sperm output can be compared to the actual daily sperm output as estimated by semen collection during the routine breeding soundness examination. If a stallion has a daily sperm output that goes below that of the predicted value for his testicular size, then he should be further evaluated for disease conditions of the testes, epididymides, and accessory glands. Testicular size is as heritable in stallions as it is in bulls [16,90,99], and therefore, stallions with measurements less than the recommended guidelines should not be used for breeding purposes.

Germinal Cell Degeneration & Renewal of Spermatogonia

In the process of spermatogenesis, spermatogonia either differentiate or remain uncommitted. This process allows spermatozoa to be produced continuously after puberty. There is a continuous production of differentiated spermatogonia that produce primary spermatocytes and ultimately spermatids. At the same time, uncommitted spermatogonia are replaced so the seminiferous epithelium doesn't become exhausted of spermatogonia and spermatogenesis can continue [57,69,72,73,79-81,84,91].

Reserve spermatogonia, which are few in number, are another population of A spermatogonia with a lifespan >60 days that do not participate in spermatogenesis but

rather serve as a source of germinal cells to repopulate the testis. In seasonally breeding males, testicular size, testosterone release, sperm production and reproductive behavior are down regulated in the nonbreeding season. Stallions outside of the breeding season maintain fertility at reduced levels whereas hamsters, deer and brown bears have near total cessation of spermatogenesis that renders them unable to breed for several months [100].

Normal spermatogenesis is a relatively inefficient process, resulting in an estimated loss of 25-75% of the potential number of mature spermatozoa produced in the adult testis. This inefficiency results from an excessive production of early germ cell types [92], that far exceed the number of germ cells the Sertoli cells are capable of caring for [101]. A study by Heninger et al. found that in normal stallions, apoptosis of germinal cells during spermatogenesis was quantified and most frequently occurred during cellular stages IV, V, and to a lesser extent VI. This quantification technique could allow future studies to compare apoptosis in normal stallions with those that have idiopathic subfertility [101].

In the stallion, testicular weight decreases in the nonbreeding season. Degeneration of germinal cells can then be estimated based on the number of spermatozoa that should be produced per gram of testis. During the breeding season, large numbers of B_2 spermatogonia are produced but 32% of the potential numbers of "young" primary spermatocytes are not formed. Sertoli cells are capable of providing for about 9-10 spermatids and two primary spermatocytes in a given generation [8,63,64] with almost all of the primary spermatocytes producing four spermatozoa [93]. In the nonbreeding season, 35% fewer A spermatogonia per gram of testis are found and 40% fewer B_2 spermatogonia [10,93,102]. The B_2 spermatogonia rise to two "young" primary

spermatocytes so the number of these cells per gram of testis is not different between seasons. About 23% of the potential numbers of spherical spermatids are not formed during the nonbreeding season. Sertoli cells are thought to be lacking in some way due to the fact that each Sertoli cell is only capable of providing for a low number of germinal cells [57,62,93]. The number of spherical spermatids converted into spermatozoa is similar between seasons. Only 75% as many spermatozoa are formed per gram of testis in the nonbreeding season as compared to the breeding season. Daily spermatozoal production in stallions is 20% lower for stallions 4-5 years old and 50% lower for stallions 6-20 years old due to differences in testis weight. Germ cell degeneration also occurs due to environmental factors such as day length or temperature and drugs among other things [57,69,72,103,104].

Leydig Cells

Leydig cells are found in the interstitial tissue and are in close proximity to blood vessels, lymphatic channels, and the basal lamina of the seminiferous tubules. Leydig cells primarily secrete steroid hormones, which aid in the function of the seminiferous epithelium, the hypothalamic-hypophyseal axis, and the accessory sex glands. Leydig cells are the site of production for most of the following: testosterone, androstenedione, androstenediol, dihydrotestosterone, 3α-androstanediol, progesterone, estrone, and estradiol. Because the hormones are produced in Leydig cells, interstitial fluid contains a much higher concentration of testosterone and other secretory products of Leydig cells than the peripheral blood (e.g., serum from blood drawn from the jugular vein). The concentration of testosterone in testis parenchyma averaged 416 ng/g and 640 ng/g in two

studies [14,91]. These values may be high due to the fact that testosterone is still produced after blood flow is stopped.

Leydig cells constantly produce small amounts of steroid hormones and are occasionally stimulated to increase testosterone production. These elevations can be 2-4 times higher and can last 2-4 h in the peripheral blood [105]. Some stallions exhibit 3-8 periodic bursts of testosterone each day. Thus, if a single blood sample is taken to evaluate testosterone levels, unusually high levels could be falsely detected.

Baseline concentrations of testosterone in the testes can be elevated >10 times during the periodic bursts. As a result, seminiferous tubules are exposed to high concentrations of testosterone constantly. Studies in other species has shown that high concentrations of testosterone is crucial for normal spermatogenesis to take place [6,69,72,82,106,107]. Minimum testosterone concentrations for normal spermatogenesis within the seminiferous epithelium are not known for stallions. Researchers have found that normal intratesticular concentrations of testosterone can be maintained by injecting rats [106,106] with massive doses of testosterone but the same cannot be done in humans and is unknown in stallions.

It was found in one study that the concentration of total 17β-hydroxy-androgen in blood leaving the testis through the testicular vein was 45 times greater than the concentration in blood taken at the same time as from the jugular vein [108]. When Leydig cells are under increased stimulation from endogenous luteinizing hormone (LH), testosterone levels in testicular vein blood can exceed 500 ng/ml. This is about a 100-fold increase from usual levels of testosterone in jugular vein blood [108,109].

Stallion Leydig cells secrete larger amounts of estrogens than testosterone but testosterone has the greatest physiologic significance. The stallion testis also secretes estrone, estradiol, estriol and two compounds called equilin and equilenin [110-115]. Aromatase is the only enzyme responsible for the irreversible bioconversion of androgens to estrogens. This enzyme is considered to be crucial and rate-limiting for the estrogen/androgen balance in the body, and thus important for estrogen-dependent processes such as bone maturation and reproduction, even in males. Thus, this enzyme has been immunolocalized in the horse testis solely in Leydig cells. The season and age-related cellular and hormonal changes observed in stallion testes should not exclude the possibility of other sources of estrogens [116]. High concentrations of free estrone and estradiol are found in testicular vein blood but most of the estrogen secreted by testis is conjugated with a sulfate (or glucuronide) side chain. This side chain restricts bioactivity and makes the compound more water soluble. It is still unclear as to the role that conjugated estrogens play in the stallion.

Leydig cells require enzymes for the production of steroid hormones of which are localized on the smooth endoplasmic reticulum and mitochondria [117,118]. In the stallions, significant correlations were found between the volume of smooth endoplasmic reticulum per testis and serum concentrations of testosterone or intratesticular testosterone content [14]. The first step in steroidogenesis involves the formation of cholesterol in two ways (1) by de novo synthesis or (2) the breakdown from low-density lipoproteins in the blood. Data based on rodent and human testes [119], have led researchers to assume that in stallion testes when minimal levels of testosterone is secreted, cholesterol for the most part is derived from intra-Leydig cell sources

(cholesterol droplets). It is also likely that when the testis is under stimulation by LH or exogenous human chorionic gonadotropin (hCG) that a majority of the cholesterol is probably derived from low-density lipoproteins in the blood. The next step in the steroidogenic pathway is the conversion of cholesterol to prenenolone, which occurs inside the mitochondria. Pregnenolone is then transported to the smooth endoplasmic reticulum where other enzymes involved in the pathway are located.

There are two general pathways that exist from pregnenolone to testosterone in stallion Leydig cells but the predominate pathway is unknown in the stallion. The first is the so-called Δ^5 pathway through dehydroepiandrosterone and Δ^5-androstenediol and the second is the Δ^4 pathway via progesterone and Δ^4-androstenedione. The Δ^4 pathway dominates in rat Leydig cells, whereas in rabbit Leydig cells the Δ^5 pathway is used almost entirely [120]. A direct and linear relationship exists between the steroidogenic enzymes that are localized on the smooth endoplasmic reticulum. This relationship holds true across species and involves the total surface area of smooth endoplasmic reticulum and its association with Leydig cells of a testis and the ability to secrete testosterone [120].

Steroidogenesis in Leydig cells is stimulated by either LH or hCG. This process is a result of LH stimulating the transport of cholesterol from intracellular stores to the outer mitochondrial membrane, and also inside the mitochondria, to provide cholesterol to the side-chain cleavage enzyme (a mitochondrial cytochrome P_{450} enzyme), which converts cholesterol to pregnenolone in a three-step sequence of reactions [118]. Cholesterol to pregnenolone is therefore the rate-limiting step in the production of testosterone. The production of pregnenolone increases with gonadotropin stimulation thus enabling an increase in the production of testosterone and estrogens.

Specific receptors for LH are located on Leydig cells and are essential to their plasma membrane. When LH binds to the membrane receptor, guanosine triphosphate (GTP-) binding protein is activated and stimulates adenylate cyclase which increases the local concentration of cyclic adenosine monophosphate (cAMP) [118]. The cAMP phosphorylates specific proteins by a posttranslational modification. A cholesterol-ester hydrolase becomes more active and releases increased amounts of free cholesterol for transport by microfilaments to mitochondria. Leydig cells rapidly increase production of testosterone in response to LH stimulation because the process does not involve synthesis of a new protein and enzymes involved in conversion of pregnenolone to testosterone are working far below their maximum capacity. LH stimulation can modify the function of other proteins to increase the transport flux of cholesterol to the inner mitochondrial membrane where the side-chain cleavage enzyme is located.

Epididymis

The specific absorptive and secretory functional aspects of each segment of the epididymis have not been as well described [28,121], as other species [24-27,122-125]. Histologically, the structure of the epididymis changes as it continues through the three different sections. The epithelial height is greatest proximally and smooth muscle components greatest distally [6,28,30]. As spermatozoa leave the testis and enter the ductuli efferentes, they are transported through several zones of the epididymis. Most of the proteins found in the epididymal fluid are secreted by the epididymal epithelium, and inhibition of this secretion leads to a loss of fertilizing ability by the spermatozoa. Each anatomical region (head, body and tail) is characterized by its own secretory activity. During transit through the epididymis, spermatozoa are bathed in various successive

biochemical environments that are specific to each region and in which sequential interactions with their membrane occur. This leads to the ability of sperm to fertilize eggs. Fouchecourt et al. completed the first study analyzing the proteins in stallion epididymal fluid. More than 250 different proteins were discovered and characterized in the fluid by 2D gel analysis. Of these 250 proteins, only 20 were abundant and represented 98% of the total secretory activity for the whole epididymis [126]. The biochemical makeup up the head of the epididymis in the stallion was found to be similar to that of the ram and boar [127].

In most mammals, spermatozoa are not mature when released from the seminiferous tubule but those taken from the tail of the epididymis are fertile [6,25,26,65,124,125,128]. Spermatozoa must undergo remodeling of the sperm membrane, such as changes in lipids, loss or modification (changes in glycosylation, proteolysis, relocalization) of preexisting (testicular) glycoproteins, and incorporation of new glycoproteins [126]. This maturation process allows the spermatozoa to gain progressive motility, structural stability, and fertilizing ability [26-28,65,103,121,122,124,125]. This maturation depends on an orderly exposure of the spermatozoa to various epididymal fluids containing enzymes and proteins that modify the plasma membrane of spermatozoa. Androgenic stimulation with testosterone is important in the head and tail of the epididymis [25,124,125]. The presence of testosterone allows for the secretion of certain proteins by the epididymal epithelium, although other secretions are produced without androgenic stimulation. The maturation process will not take place if spermatozoa are simply retained in a given segment [122,124,125].

Stallion spermatozoa from the head or proximal body of the epididymis are immotile when released into a physiologic salt solution. Samples removed from the tail and diluted in buffer are similar in motility to spermatozoa collected in ejaculated from the same stallions. Hence, stallion spermatozoa are mature before entering the tail of the epididymis if progressive motility is used as a measurement of maturity [28]. It has been shown that spermatozoa from all regions of the epididymis are resistant to "cold shock" whereas ejaculated spermatozoa are altered by rapid cooling to 0°C. Based on these and other observations, Johnson et al. concluded that maturation of spermatozoa in the stallion was not completed until spermatozoa left the tail of the epididymis [28]. This is consistent with data for other species [25,27,124,125], although in rams, changes in spermatozoa that result in a greater percentage of surviving embryos occur in the tail of the epididymis [25]. In 1957, a mare artificially inseminated and became pregnant with frozen epididymal spermatozoa [129]. In a more recent study by Bruemmer, it was shown that spermatozoa capable of fertilization could be removed from the tail of the epididymis following a catastrophic injury, death, or even elective castration. Most samples obtained from these stallions provided 5 to 25 breeding doses [130].

Although spermatozoa are found throughout the epididymis [24,59,60,131,132], the tail of the epididymis and deferent duct (including the ampulla) are the major spermatozoal storage areas. The two tails of the epididymis of a normal, sexually rested adult stallion (5-16 years old) should contain about 54 billion spermatozoa, or approximately 61% of the total sperm in the excurrent duct system [130,131]. In stallions, the tail of the epididymis contain more spermatozoa than the tail from a bull, but a much lower number than the tail from a ram or boar. Sufficient numbers of spermatozoa are present within

the tail of the epididymis in the horse for several ejaculates [60,131]. Testis size, daily spermatozoal production, and number of spermatozoa stored within the epididymis are influenced by age.

Spermatozoa do not swim through the epididymis and deferent duct. Movement of spermatozoa through the epididymal duct is primarily by continuous peristaltic contractions of smooth muscle in the wall of the duct within the head and body of the epididymis. In the body, the epididymal duct normally is inactive, except when smooth muscle is stimulated to contract. Consequently, time required for movement of spermatozoa through the head and body of the epididymis is not altered by ejaculation and averages about 4.1 days in stallions [59,60,131,132].

Fertility of spermatozoa is usually not depressed in males ejaculating frequently, because rate of spermatozoal transport through the caput and corpus epididymidis is not influenced by ejaculation; decreased fertility could result if number of spermatozoa ejaculated were less than the number required for maximum reproductive efficiency. In a study with a small number of sexually rested stallions, five to seven consecutive days of ejaculation were required to deplete extragonadal reserves. It was suggested that with daily collections, the number of spermatozoa collected on the seventh day will approximate daily spermatozoal output [133], while another report suggested that the precision of this single-day estimation procedure was very low, but was improved by increasing the number of daily ejaculates evaluated [88]. Extensive data for bulls for bulls ejaculating daily, or at a similar high frequency, show that spermatozoal fertility is equivalent, if not slightly superior, to that for bulls ejaculating once a week [134-136]. When seven successive ejaculates were collected from beef bulls and the spermatozoa

were used to artificially inseminate cattle, fertility did not differ among the seven successive ejaculates [137]. Fertility of stallion spermatozoa used for artificial insemination should be similar whether stallions are collected daily, every other day, or every 4 days.

The interval that spermatozoa spend in the cauda epididymidis is influenced by ejaculation [60,131]. The number of spermatozoa in the cauda epididymidis is maximal in sexually rested stallions and is reduced in males ejaculating daily or every other day [131]. Because fewer spermatozoa are present in the cauda epididymidis of a stallion ejaculating regularly than in an inactive male, transit time for spermatozoa through the cauda epididymidis of a sexually active stallion is reduced by 2 or 3 days from the 10 days characteristic of a sexually rested stallion [59,131,132]. Although the time required for movement of spermatozoa through the caput and corpus epididymidis is reasonably similar among species [60], the interval that spermatozoa spend in the cauda epididymidis differs greatly, and ranges from < 4 days in humans and some beef bulls to > 12 days in rams.

Spermatozoa are produced continuously, regardless of ejaculation frequency. Because spermatozoa enter the epididymis at a constant rate, they must also leave the excurrent duct system at a relatively constant rate, although this rate is altered by ejaculation. Based on research with several species [138,139], all spermatozoa that enter the excurrent duct system of a stallion likely leave through the urethra. Resorption of spermatozoa probably does not occur within the excurrent duct system [60,138,139]. In bulls and rams, spermatozoa that are not ejaculated at copulation or voided by masturbation are eliminated periodically during urination [139]. Probably in a normal, sexually inactive

stallion, spermatozoa intermittently pass from the deferent duct into the pelvic urethra and are voided during urination. The direct cause for or interval between such emissions is unknown. Certain stallions accumulate an abnormally large number of spermatozoa in the epididymis and perhaps to some extent in the deferent duct, including the ampulla. In such a horse, spontaneous emissions probably do not occur and spermatozoa accumulate in the epididymis until the limit of distensibility of the epididymal duct is reached. As a consequence of this accumulation, storage interval of spermatozoa in the cauda epididymidis of such a stallion is much longer than 7 to 10 days, and spermatozoa may undergo marked alterations. Much lower percentages of spermatozoa are structurally normal or motile in the first several ejaculates collected from a stallion accumulating spermatozoa than in a normal horse because of the prolonged storage interval.

Accessory Sex Glands

The prostate gland, vesicular glands, bulbourethral glands, and ampullae are collectively referred to as the accessory sex glands. A bulk of the ejaculate volume is composed of seminal plasma from the secretions of the accessory sex glands. Spermatozoa from the cauda epididymidis and deferent duct are immotile until mixed with accessory sex gland fluids at ejaculation (or mixed with a buffer by human intervention). Exact factors causing initiation of motility are unknown, but intracellular pH may be involved. Seminal plasma plays an important role in sperm function [76,140,141] but exposure to these fluids is not necessary for normal fertility of spermatozoa. Evidence in some studies has shown that long-term exposure may decrease sperm motility and increase sperm death during semen storage [142-146]. Breeding programs remove seminal plasma when semen is frozen. Before cooled semen storage, seminal plasma is either

removed by centrifugation or its influence is reduced by dilution with an extender [142]. Webb and Arns found that the removal of seminal plasma and the dilution of sperm with skim milk extender containing modified Tyrode's medium (KMT) improved motion characteristics during cooled storage of spermatozoa [147]. KMT was detrimental to sperm motility in the presence of seminal plasma [148].

The addition of seminal plasma to sperm recovered from the epididymis has been shown to reduce the freezability of the sperm [144]. On the other hand, stallions that do not retain adequate fertility after semen thawing can benefit from the addition of seminal plasma from a stallion with good fertility. This seminal plasma can improve the motility and membrane integrity of the frozen semen post thawing [149]. In contrast, Akcay et al. found that seminal plasma taken from a stallion with high motility lowered the progressive motility and viability of sperm when added to a stallion with low motility [142]. The proteins in seminal plasma of fertile and subfertile stallions has been characterized and compared. Subfertile stallions were shown to have increased concentrations of certain specific proteins [76,149,150]. Amann et al. demonstrated a variation in several of the seminal plasma proteins with post-thaw motility of cryopreserved sperm but the protein levels did not adequately predict a stallion's response to sperm freezing [143]. Seminal plasma was shown to suppress the inflammatory response of the mare's endometrium to sperm following insemination or natural mating [141,151]. Although the specific functions of seminal plasma are not known, it does provide transport of ejaculated sperm from the stallion's tract, energy for the sperm, in addition to a protein source and other macromolecules necessary for sperm function and metabolism [6,29,143].

Normal function of all accessory sex glands depends on availability of testosterone in peripheral blood [152]. The secretion of the prostate gland is thin and watery [152,153]. This secretion probably helps cleanse the urethra during ejaculation and also constitutes a major portion of seminal plasma, especially if a second ejaculation occurs 1 to 3 h after an earlier ejaculation. Depending on season and individual stallion, fluid secreted by the vesicular glands may (or may not) contribute a major portion of seminal plasma in an ejaculate. The gelatinous material often found in seminal plasma, especially in April to July is secreted by these glands [153]. This seasonal change in secretion of gel by vesicular glands, as well as the stallion-to-stallion difference, may reflect differences in concentration of testosterone in blood. Two bulbourethral glands are positioned on either side of the pelvic urethra near the ischial arch. Their secretion contributes to the seminal plasma but probably only a minor portion in terms of volume.

Endocrine Hormones

The neuroendocrine system controls the function of reproductive organs. This system includes specialized nerve cell bodies and endocrine tissues that secrete chemical messengers termed hormones. The hormones are then carried through the blood from one organ to control the function of another organ. The autonomic nervous system also plays a role in controlling the function of the reproductive organs. It plays an integral part in transporting spermatozoa from the testes through the epididymides and deferent ducts and also in erection, emission, and ejaculation. Maintenance of normal function of reproductive organs depends mainly on the neuroendocrine system.

Hypothalamic-Hypophyseal Axis

The hypothalamus is part of the diencephalon of the brain and is involved in numerous functions including; regulation of appetite and thirst, body temperature, vasomotor activity, emotion, use of body nutrient reserves, activity of the intestinal tract and bladder, states of sleep and wakefulness, sexual behavior, and release of tropic hormones.

The hypophysis is connected to the hypothalamus and extends downward from it. A number of hormones that control reproductive processes in the stallion and mare are synthesized and discharged from the adenohypophysis [154,155]. The neurohypophysis serves as a storage reservoir for hormones produced by neural tissue within the brain but doesn't produce any hormones itself. Portal vessels extend from the hypothalamus through the infundibulum to the pars distalis thus linking the hypothalamus and adenohypophysis. Blood within the portal system normally flows directly from the hypothalamus to the pars distalis and is the only direct link between the two.

The hypothalamus synthesizes and discharges a number of "releasing hormones" with proper neural stimulation. Gonadotropin-releasing hormone (GnRH) is directly involved in controlling reproductive function and is discharged by the hypothalamus in short, pulsatile bursts. GnRH is rapidly removed from the blood resulting in pulsatile stimulation of the gonadotropin-secreting cells in the adenohypophysis. The synthesis of structural analogues of GnRH has made it possible for research involving the study and control of reproductive function. Two types of analogues of GnRH are available (1) those that block the action of the natural hormone and (2) those that are more effective in inducing a response than natural GnRH.

At least six tropic hormones are produced by the adenohypophysis although only two or three have a direct role in male reproduction. LH and FSH are gonadotropic hormones that are produced by the adenohypophysis in direct response to stimulation by GnRH. They are termed gonadotropic hormones because they act on the gonads to stimulate their function, including the production of spermatozoa and steroid hormones. As observed in other species, under appropriate stimuli, the hypothalamus of the stallions releases GnRH in a pulsatile fashion which in turn stimulates the production and episodic secretion of LH and FSH [156-160]. In stallions, LH release is positively correlated with day length, with LH concentrations rising in the beginning of the breeding season [100]. Basal LH concentrations in the summer are twice as high as near the winter season [100]. In some stallions during the breeding season, pulsatile secretion of LH is not apparent from analyses of jugular blood [105,159]. There are conflicting reports on seasonal changes of FSH in the stallion. Mean plasma FSH concentrations in stallions are relatively constant throughout the year and only minor increases during the breeding season have been reported [100]. Roser et al. found that in the stallion, FSH and inhibin exhibit similar seasonal changes; peripheral FSH and inhibin increase in the spring and decrease in the fall [161]. FSH is secreted in a pulsatile manner, although more short-term inconsistency exists [105,159], perhaps reflecting sporadic discharges of small amounts of hormone. LH secretion often coincides with pulsatile secretion of FSH. Several stallion studies have analyzed the concentrations of hormones in blood [63,103,105,114,152,156,157,159,160,162-166], but concepts for endocrine control of reproductive function are based primarily on data from other species [118,154,167-169]. Seasonal changes in the GnRH/LH release in the horse are partly regulated by changes in

a GnRH-inhibitory opioidergic tone. An acute LH release in stallions could be induced with the opioid antagonist naloxone outside of, but not during the breeding season [170]. This indicates that opioidergic neuronal systems in the stallion, as found in mares [171], inhibit pulsatile GnRH release outside the breeding season and are partly responsible for reduced LH release and decrease in testicular function during the winter months [172].

Testicular Hormones

Leydig cells produce steroid hormones which are the primary endocrine product of the testes. Sertoli cells secrete two glycoproteins called inhibin and activin which share a common subunit [173]. The inhibin/activin family consists of three subunits: α, β_A and β_B. Inhibin is the combination α/β_A or α/β_B, and activin is the combinations β_A/β_B, β_A/β_A, or β_B/β_B. The amounts of different inhibins, or activins, secreted fluctuate as a function of cell type (Sertoli cells of male or granulosa cells of female, species, and stage of development). Sertoli cells in the stallion secrete inhibin and activin of which the exact molecular forms of each are still being established.

The exact role of oxytocin in the male animal is not clear, but oxytocin treatments shortly before ejaculation increases the number of sperm in the ejaculate of the bull, ram, rabbit, and rat [174]. It is suggested that this effect is mediated via a strong contractile response in the testes and excurrent ducts, which increases sperm transport, and it has been shown in vitro that contractile activity of seminiferous tubules is reduced in the absence of oxytocin [174]. Watson et al. were the first to find oxytocin in gonadal tissue and in semen from stallions. The concentration of oxytocin in seminal fluid was similar to that measured in seminal fluid from men. It was also shown that the testis of the stallion does not synthesize oxytocin and that the epididymis also contains oxytocin that most likely

plays a role in smooth muscle contractility and sperm transport in the stallion. Oxytocin is highly associated with the gel fraction in stallions suggesting that concentrations in semen arise from either the peripheral circulation or from the vesicular glands [174]. Studies found that Leydig cells in rats and rams likely secrete oxytocin into the interstitial fluid [175,176]. It is likely that oxytocin is transported by Sertoli cells into the luminal fluid or possibly secreted by the rete testis. When evaluating the rete testis fluid of rams, it was found that the fluid contained about 550 pg/ml as compared with 124 pg/ml in serum from testicular venous blood [175]. Veeramachaneni and Amann contemplated that sperm stasis and formation of granulomatous lesions (common in cattle, sheep, and goats, but frequency in horses is unknown) could possibly be due to a deficiency of testicular oxytocin in the fluid that enters the excurrent ducts or is taken up by the epithelium [177].

Regulation of Hormone Secretion

Production of testosterone in the adult male is controlled by episodic bursts of LH secretion, which occasionally elevate the concentration of LH in blood reaching the testis above the baseline level. Therefore, basal production of testosterone is amplified by episodic bursts of production of testosterone [103,108,112,156,159,163].

The testosterone that Leydig cells produce enters the venous blood draining from the testis, passes through general circulation, and the moves to the hypothalamus and adenohypophysis. Through this long feedback loop, testosterone mediates the discharge of GnRH and LH [152,157]. If testosterone concentrations at the hypothalamus and adenohypophysis are high, release of GnRH by the hypothalamus is held back and the adenohypophysis doesn't react to accessible levels of GnRH. Additionally, the target

cells of the hypothalamus or adenohypophysis may possibly convert testosterone to estradiol. Due to this negative feedback, LH concentrations would be low in blood entering the testis, therefore Leydig cell exposure to LH is low resulting in testosterone being secreted by the Leydig cells at a basal rate. As testosterone concentrations in peripheral blood decline, the negative block is removed and GnRH is released intermittently from the hypothalamus. LH is then released from the adenohypophysis, resulting in an elevated concentration of LH in the blood flowing to the testis, and rapid stimulation of Leydig cells to produce and discharge testosterone. A circular feedback loop is formed between Leydig cells, the hypothalamus, and the adenohypophysis ultimately regulating concentrations of LH and testosterone in peripheral blood.

The mechanism by which FSH secretion is stimulated by GnRH is different than that of LH. Bursts of LH are not always accompanied by a release of FSH and vice versa [105,159]. FSH synthesis and secretion appear to be less dependent on GnRH than that of LH [6]. Research with non-equine species showed that FSH acts entirely on Sertoli cells within the seminiferous tubules [6,167]. Sertoli cells produce several protein hormones, including closely related inhibin and activin. Along with other functions, these hormones act on the adenohypophysis to suppress (inhibin) or stimulate (activin) secretion of FSH, with little to no effect on the secretion of LH. In stallions, as well as in rams, concentrations of inhibin in plasma vary according to seasonal reproductive state and are positively correlated with testicular size and testosterone secretion [100]. The relative amounts of LH and FSH secreted in response to GnRH from the hypothalamus are probably controlled by the inhibin/activin system and the ratio of testosterone to estradiol impinging on the adenohypophysis.

Stallions have a uniquely high concentration of estrogens in their testes. Estrogens synthesized in the testis of mammals and detected in ejaculate appear to come from Leydig cells [178]. These hormones have a role in the autocrine control of testosterone production and act within the seminiferous tubules. In addition, they also regulate the resorption of luminal fluid in the head of the epididymis, making them essential hormones for the male reproductive system [179]. It is hypothesized that estradiol or other estrogens are transformed within Leydig cells from a portion of testosterone. High levels of estrogens can be found in the blood draining from the stallion testis. These high concentrations of estrogens flowing to the hypothalamus and adenohypophysis may suppress discharge of GnRH or LH and FSH in a negative feedback loop [152,180]. Injecting hormones in stallions can alter the hormonal balance because of feedback loops involving the hypothalamus, adenohypophysis, and testis. Reproductive function can be disturbed with sequelae of such manipulations often having undesirable results.

Hormonal Control of Spermatogenesis

The hormonal requirements for normal spermatogenesis in a stallion are not entirely known. The duration of the cycle of seminiferous epithelium and total duration of spermatogenesis are most likely not modified by hormonal balance within the testis of rats [69,72,80,84]. Hormones partially control the degree of germ cell degeneration [72,80,82,84,181]. Leydig cells produce testosterone and surround the seminiferous epithelium, thus exposing it to a higher concentration of testosterone that the peripheral blood. Normal spermatogenesis requires high levels of testosterone [82,181], whereas in rams, FSH is necessary for spermatogenesis [62,65,82,181], and LH may have a direct role in regulating division of spermatogonia in addition to its role at stimulating

testosterone production [181]. In contrast, spermatogenesis can be maintained by testosterone alone in rats [65]. Sertoli cells mediate the role of FSH in the normal development of spermatids [62]. Hormonal control of spermatogenesis in stallions is still under much debate, because some differences among species exist [181].

Descent of the Testes

In the normal colt, both testes should descend into the scrotum between 30 days before and 10 days after birth [17,182,183]. Androgen production by the developing fetal gonads probably plays an important role, as may mullerian inhibiting factor and epidermal growth factor [5,184].

By day 40 of gestation, the testis is suspended from the abdominal wall and the mesonephric duct, which later gives rise to the epididymis and ductus deferens, leads into the pelvic area [182]. A narrow invagination, termed the vaginal process, starts to form about day 43 of gestation and progressively develops to form the inguinal canal. Around day 150, the developing cauda epididymis is drawn to or just within the internal inguinal ring, but the testis is large and cannot enter in the inguinal canal [182]. Entrance of the testis into the inguinal canal typically begins between 270 and 300 days of gestation [182]. This occurs only after the vaginal process and internal inguinal ring have been stretched sufficiently by enlarging cauda epididymidis to allow entrance of the testis that has diminished in size from 50 to 30 g. Pressure from fluid in the abdominal cavity, and possibly from the intestines, forces the testis down through the inguinal canal. This descent places the lamina visceralis or the tunica vaginalis, the outer covering of the testis, into apposition with the lamina parietalis of the tunica vaginalis, the former vaginal process, separated by the cavum vaginale.

Failure of normal testicular descent is common in horses [17] and is termed cryptorchidism. The left testis is more commonly retained in stallions [17]. The undescended testis is exposed to higher temperatures and therefore spermatogenesis is often disrupted at the time of puberty. In the event of a unilateral cryptorchid, the stallion may still be fertile but since the condition is often hereditary, castration should be performed. Diagnosis of cryptorchidism is attained by manual palpation of scrotal content in addition to rectal palpation and careful inguinal palpation to assist in identification of an abdominally or inguinally retained testis. Ultrasonography has been recommended as a diagnostic tool for this as well [185]. In horses with bilaterally retained testicles or apparent geldings with stallion-like behavior, hormonal profiles may be useful in diagnosis of retained testes [186,187]. Baseline testosterone levels have been suggested as a method to diagnose retained testicular tissue in an apparent gelding [186,188]. Numerous problems are associated with the test including low wintertime testosterone values in normal stallions, a relatively high percentage of nondiagnostic values, and false negative values. The use of a single measurement of plasma conjugated estrogens, without human chorionic gonadotropin stimulation also appears to be reliable in the diagnosis of cryptorchidism in colts older than 3 years of age [186]. A stimulation test using human chorionic gonadotropin reduces the number of nondiagnostic test results obtained with both conjugated estrogens and testosterone measurement. In the test, 5000 to 10,000 IU of human chorionic gonadotropin is injected intravenously. Blood samples for conjugated estrogens and testosterone are obtained before the injection and 60 to 120 minutes later. A fivefold or greater increase in hormone (conjugated estrogens or testosterone) indicates that a retained testicle is present. One study demonstrated that the

increase in conjugate estrogen and testosterone after human chorionic gonadotropin stimulation peaked 2 to 3 days after the injection [187]. Until a colt is older than 3 years of age, false-negative results may still occur whether testosterone or conjugated estrogens are measured [186,189].

Bergin et al. reported that the earliest complete descent of both testes was at 315 days of gestation, about 25 days before parturition [182]. In 32 fetuses between 9 months of gestation and birth, descent of the right testis was more advanced than the left in 78% of the fetuses, while the left testis was more advanced in only 3%. Of 12 fetuses collected at term, 42% had completely descended testes, 25% had both testes within the inguinal canals, 17% had one testis in the scrotum and one in the inguinal canal, and 17% had both testes within the abdominal cavity. A total of 5 of 9 colts less than 1 week old had complete bilateral descent of the testes into the scrotum.

As reviewed by Bergin et al. failure of the testes to descend has been attributed to abnormalities of the testis, development of adhesions between the testis and adjacent structures, or an abnormal outpouching of the vaginal process [182]. Bergin et al. discounted these factors as causes of cryptorchidism and suggested that the most obvious reasons for the testis to remain in the abdominal cavity included the following: insufficient abdominal pressure to properly expand the vaginal process; stretching of the gubernacular cord; insufficient growth of the gubernaculum and cauda epididymidis so that they are unable to expand the inguinal ring sufficiently to allow entrance of the testis.; and displacement of the testis to a position where intestinal pressure prevents tension from the gubernaculum, via the gubernacular cord, pulling the testis into the vaginal process.

Puberty

The testis contains few (if any) functional Leydig cells and only indifferent supporting cells and gonocytes (progenitors of Sertoli cells and spermatogonia) at birth. The infantile stage of the stallion's life then begins. Data from other species describes this infantile stage by a declining number of gonocytes, absence of gonadotropin secretion by the adenohypophysis, and limited steroidogenesis (at least in terms of testosterone) by Leydig cells [82,190-194]. Spermatozoa are not produced during the infantile stage which continues through ≥ 6 months in stallions, and changes are initiated which continue through a prepubertal stage and culminate in puberty. Breed and season of birth can influence the timing of the prepubertal changes among stallions. Puberty is defined as the time when a stallion is first capable of successfully participating in reproduction. Domestic animal literature states that puberty is considered to be a definitive end point. A period when the animal is capable of reproduction and, by definition, implies achievement of spermatozoa production and completion of prepubertal events. In human literature, the term puberty has no specific end point, but refers rather to the series of events termed the prepubertal stage. Some stallions develop a few spermatozoa that are available for ejaculation by 14 months of age. Following puberty, development of reproductive capacity continues (postpubertal stage), and 2-4 yr after puberty, a stallion achieves sexual maturity (maximum reproductive capacity). Years later, reproductive senescence may occur but for most stallions, no change in daily spermatozoal production occurs between 4-20 yr of age [64].

Research is lacking regarding testicular function or changes in the neuroendocrine system of stallions during the infantile stage of development or exactly when the prepubertal

stage is initiated. Light horse and draft breeds vary in timing of specific events. Around 9 months of age, concentrations of FSH and LH in blood increase then around 12 months, the testes start to develop and grow rapidly [164,195]. A few months following that, spermatozoa are gradually produced [164,196]. There is a linear increase in total scrotal width from 42 mm at 42 weeks to 85 mm at 96 weeks of age (about 1 mm/week), based on a study of 15 colts born in July and early August [164]. After 80 weeks of age, increased levels of testosterone can be found in jugular vein blood [164]. These prepubertal developments are concluded in puberty when a stallion produces spermatozoa and would be fertile if allowed to breed a mare.

The study carefully monitored 15 colts born in July and early August for reproductive development, and discovered their age at puberty averaged 83 weeks (puberty defined as first ejaculate containing 50 million spermatozoa of which ≥ 10% are motile) [164]. Puberty was reached by 90 weeks of age in 11 of the 15 colts and following this period, there was a slow increase in both quantity and quality of spermatozoa. The percentages of motile and structurally normal spermatozoa were still low at 2 yr of age, but the ejaculates contained 3.3 billion spermatozoa. This contrasts with bulls in that the fertility of spermatozoa increases for about 6 months after puberty.

Changes in the hypothalamic-hypophyseal-testicular axis of the stallion are based on information for bulls, rams, and other species due to a lack of information [168,169,190-194]. During the infantile stage, LH, FSH and testosterone levels are secreted at low levels. As the transition from the infantile stage to the prepubertal stage takes place, an increase in the frequency and amplitude of LH discharges occurs [168,169,190,192]. Before about 36 weeks of age stallions have a relatively low secretion rate of LH which

most likely reflects a low concentration of GnRH receptors in adenohypophysis [192] and little secretion of GnRH from the hypothalamus. LH secretion is high in bulls and is first evidenced by frequent discharges of high amplitude starting around 10-12 weeks of age continuing for about 6 weeks [190]. This event is similar in stallions, as verified by the high concentrations of LH in jugular-vein blood between 36-45 weeks of age. It is thought that in stallions, the surge of LH secretion may simply be a change in amplitude of the LH pulses rather than a combined effect of increased frequency and volume of LH with each discharge [105,117,156]. The seasonal rise of serum concentration of LH in adult stallions is exclusively caused by an increase in LH pulse amplitude [105,117,156]. The stallion hypophysis has the capability to secrete considerably more LH at 32 weeks of age than actually is secreted [165], although after GnRH administration LH release increased from 32-48 weeks of age.

Naden et al. found that in eight stallions, an increase in LH-stimulation which occurred from 36-45 weeks of age did not affect Leydig cells because there was no change in testosterone concentration as evidenced from peripheral blood samples [164]. Data available on bulls showed the same occurrence [168,169,190,192]. It is likely that Leydig cells require prolonged stimulation by LH to complete their differentiation and develop the ability to secrete testosterone. It takes nearly twelve months in stallions between the initial increase of LH concentration in the blood and an increase of testosterone concentration [164], compared to three months in the bull [168,169,190]. This difference could be a consequence of a more pronounced seasonal effect on reproductive function in stallions than in bulls. In addition, Naden's study exposed Leydig cells to high concentrations of LH from May to July, leading to a seasonal effect

three months later that possibly suppressed secretion of testosterone that might have occurred [164]. In the stallion during the nonbreeding season (September-July), secretion of testosterone is decreased and blood concentrations are low [164]. The secretion of LH, FSH, and testosterone all increased during the next breeding season in the young colts born in July through early August.

Reasons for inactivity of the neuroendocrine system during the infantile stage are still unknown in the stallion but data from bulls and rams have been examined [168,169,181,190-192]. It is hypothesized that in stallions an estradiol-mediated block suppresses secretion of GnRH by the hypothalamus before about 16 weeks of age. If neurochemical stimuli are present, pulsatile discharges of GnRH begin or increase. At the same time, an increase in the concentration of estradiol receptors in the adenohypophysis may allow initiation of positive feedback of estradiol on this organ. An increase in the concentration of GnRH receptors in the adenohypophysis ensues and there is an increased responsiveness of the adenohypophysis to discharges of GnRH initiated. Following these events, LH is secreted in limited amounts by 16 weeks of age. GnRH continues to stimulate the adenohypophysis thus increasing the synthesis of mRNA for the β-subunit of LH between 20-32 weeks of age. The continued stimulation by GnRH enables copious production of LH from 32-45 weeks of age.

The study by Naden et al. also considered that the maturation of the hypothalamic-hypophyseal axis might be estradiol or androstenedione induced, rather than testosterone [164]. It was thought that the cause of the seasonal suppression in LH secretion from 55-85 weeks of age in colts studied was due to the secretion of a gonadal steroid, other than testosterone, acting on the hypothalamic-hypophyseal axis [164]. For species other than

stallions, the support of spermatogenesis relies on supporting cells being exposed to FSH and receiving androgenic stimulation to complete their differentiation into Sertoli cells. The data show that there is sufficient androgenic stimulation provided by the intratesticular environment at 70-75 weeks of age enabling the onset of spermatogenesis and ejaculation of spermatozoa at 83 weeks of age (puberty) while the secretion of testosterone increased for at least several months after the initial rise around 72 weeks of age. When comparing stallions to available data for bulls and rats [60,193,194], the efficiency of spermatozoal production in stallions probably increases rapidly through the interval from puberty to > 100 weeks of age.

Ejaculation

Three sequential processes: (1) erection, (2) emission, and (3) ejaculation - are involved in the process of ejaculation. Erection occurs when blood engorges the corpus cavernosum and corpus spongiosum penis resulting in the lengthening and stiffening of the penis. Emission is the movement and deposition of spermatozoa and fluid from the ductus deferens and cauda epididymis, and fluids from the accessory sex glands, into the pelvic urethra. Ejaculation is the actual discharge of semen through the urethra.

An erection can be initiated either by sensory stimuli of the glans penis or by psychic stimulation of the cerebral cortex. The cerebrum reacts to visual stimuli during teasing thus resulting in an erection. Parasympathetic impulses pass from the second, third, and fourth sacral ligaments of the spinal cord, via splanchnic nerves, to the penis. These impulses override sympathetic stimulation which normally keeps arterioles in the penis partly constricted thus allowing dilation of the penile arterioles. Blood enters the corpus cavernosum and corpus spongiosum penis while at the same time, extrinsic muscles of

the penis (i.e., ischiocavernosus, bulbospongiosus, and urethralis) which contract and compress the deep and dorsal veins of the penis against the ischial arch. This impedes the venous return from the cavernous bodies of the penis. Blood is then shunted to fill and distend the corpus cavernosum and corpus spongiosum penis resulting in enlargement of the penis. The penis returns to its flaccid state when the arteries constrict to their normal state as a result of sympathetic impulses, and pressure on the veins is relieved by relaxation of the ischiocavernosus and bulbospongiosus muscles.

In the stallion, emission and ejaculation occur as a series of strong, pulsatile contractions of the urethralis and bulbospongiosus muscles so that several successive jets of semen are ejaculated [197]. Weber and Woods were the first researchers to use transrectal ultrasonography to evaluate the accessory sex glands in ejaculating stallions. Their study confirmed that the pattern of seminal emission is consistent with the sequence in which accessory sex gland secretions appear in stallions ejaculates, with the highest spermatozoa concentration in the first seminal fraction and decreasing concentrations in successive fractions [198]. First, the prostate gland secretes a watery fluid into the pelvic urethra and some prostatic fluid is ejaculated as a prespermatozoal fraction. Next, emission and ejaculation of the spermatozoal-rich fraction occurs. This fraction consists of spermatozoa and epididymal secretions and probably prostatic fluids and watery bulbourethral gland fluid. Usually, three to six sequential discharges of sperm-rich fluid occur. The vesicular glands release the gel or postspermatozoal fraction. The gel is significantly decreased if a second ejaculate is taken in a 2 h period. Semen characteristics differ depending on season, age, testicular size, interval of abstinence

since the previous ejaculation, and the extent of sexual arousal or courtship before ejaculation.

PART II

Testicular Degeneration in Stallions

The process of spermatogenesis is very fragile, extensive and easily upset by a variety of extrinsic factors [199]. The susceptibility of the germinal epithelium to damage makes testicular degeneration (TD) a common cause of acquired, progressive infertility and often sterility in stallions [200-202]. TD is a poorly understood disease in males with a significant economic impact, especially in stallions. TD results in poor semen quality characterized by a high percentage of morphologic spermatozoal defects, poor motility, a low number of normal sperm per ejaculate and decreased testicular size. TD can affect one or both testes, depending on whether the contributing reason is localized (focal lesion), as with a locally aggressive tumor, or generalized (diffuse lesion), as with fever of variable origin or ITD [202,203]. TD can manifest in two ways, 1) acutely and with a known insult on the testis or 2) for reasons unknown. TD has a numerous causes with thermal injury being the most common. Any event that disturbs the vital temperature differential of the testis can interfere with normal spermatogenic function. Insults that can be identified that can result in TD in the stallion include, high environmental temperature, fever, orchitis, periorchitis, hydrocele, scrotal hemorrhage, scrotal edema, scrotal dermatitis, improper scrotal descent, systemic/local infections, injury to essential vasculature (torsion), inflammation of the testicular artery or degenerative changes in testicular arterioles, hormonal disturbances, ionizing radiation, malnutrition, ingestion of toxic plants, neoplasia, efferent/epididymal duct obstruction, production of antisperm

antibodies or uncontrolled intratesticular hemorrhage [203-207]. Though the germinal epithelium is quite susceptible to injury, damage is often temporary because of the more resistant nature of the stem-cell spermatogonia, Sertoli cells and Leydig cells. These cells allow restoration of normal spermatogenic function if the contributing agent for the TD is removed. TD can be reversible depending on the duration and severity of the insult. Mild cases of TD are able to return to full function whereas in severe cases of TD, the affected testes usually do not progress once the insult has been removed but return to function is unknown. Cases of TD in which no cause can be identified is referred to as idiopathic testicular degeneration (ITD) [206,208]. In infertile men, only 25% have defined conditions [209]. ITD is often age-related and is found in middle-older age stallions but it can affect much younger animals too [206]. ITD is extremely progressive in nature, beginning with a reduction in fertility and possibly leading to sterility. Several researchers have reported differences in plasma hormone concentration and Leydig cell morphology in stallions with ITD compared to stallions with androgen or heat induced TD [204,210,211]. This leads some researches to consider ITD as a separate condition from TD.

A majority of the studies concerning ITD has been done in rats. These studies have shown that age-related declines in testicular function are likely due to the testis and not defects in the hypothalamic-pituitary axis [212]. The number of Leydig cells does not change as the testis ages. Old Leydig cells have been shown to contain less LH receptors when compared to younger Leydig cells. As the rat ages, the Leydig cells become less efficient at producing testosterone and FSH levels increase, thus the signaling pathways and steps in the steroid pathway become impaired in the Leydig cells [213].

Additionally, a study looked at gene expression in rat Leydig cells during their development and found gene profiling demonstrates that postnatal development is associated with changes in the expression levels of several different clusters of genes consistent with the processes of Leydig cell growth and differentiation [214]. It has been hypothesized that during normal spermatogenesis, reactive oxygen products are produced and thus over time cause damage to Leydig cells.

Several studies have attempted to determine the causes of ITD in stallions [85,215-217]. These studies determined that the endocrine changes in aging, subfertile stallions varied significantly depending on the severity of the problem and the reason for subfertility. A comparison study was done on plasma hormone concentrations between normal, fertile animals and subfertile animals with mild testicular changes, revealing no significant differences amongst the two groups. More severely affected subfertile/infertile animals showed elevated levels of LH and FSH in plasma, decreased plasma levels of inhibin and estradiol and decreased intratesticular inhibin concentrations. Elevated levels of FSH in the plasma combined with low plasma estrogens in the face of low fertility have been implicated in cases of TD [208,215]. Like the rat, studies testing hypothalamic, pituitary and testicular function in fertile, subfertile and infertile stallions indicate that the primary problem in idiopathic stallion infertility lies in the testis itself and not in the hypothalamus or pituitary [216,218].

LH receptors were evaluated and compared in the testes of subfertile and normal stallions. Unlike the rat, there was no difference in the number of receptors between the two groups [219]. This leads researchers to believe the problem is not in the LH receptor number but possibly in the steroidogenic pathway itself [218]. One of the first changes in

steroid levels in subfertile stallions is decreased levels of testicular inhibin. Therefore the defect may reside in the Sertoli cell rather than the Leydig cell in cases of idiopathic infertility [217], providing further evidence that the primary cause of ITD resides at the level of the testis and not the hypothalamus or pituitary.

Turner and Casas-Dolz [220] looked at differences in gene expression in the testes of a fertile stallion compared to the testes of one young stallion and one old stallion diagnosed with severe ITD. They suspected that multiple genes are involved in ITD since it has many characteristics of a multigenic condition (e.g., inconsistent penetrance, stages of severity, high prevalence). Their results found few differences in gene expression between the ITD stallions and the fertile stallion [220]. In addition, several genes were found to be differentially expressed (either up or down regulated) when comparing the fertile stallion to one of the ITD stallions but findings were not consistent in both [220]. These findings indicate that ITD truly encompasses an assorted collection of problems and that the function of these genes in the pathogenesis of ITD is still unclear.

In a study by Blanchard and Johnson [221], there was an increased degeneration of germ cells in stallions that produced low sperm numbers. This degeneration was evident during early meiosis and spermatogenesis in addition to having lower germ cell:Sertoli cell ratios. When the stages of cells were closely evaluated, it appeared that earlier stage germ cells (e.g., spermatogonial stem cells) and testicular somatic cells (Leydig and Sertoli cells) were more resilient to degenerative changes. Therefore, the remaining germ cells may have the ability to restore normal spermatogenesis if the inciting cause of TD can be identified and removed. Normal spermatogenesis often resumes in cases of TD in

which the insult is identified and removed unlike ITD which isn't reversible and often progresses in severity.

In 2005, Dobrinski performed the first study in which testicular tissue xenografts were taken from neonatal pigs and goats and then placed in castrated and immunocompromised mice. These xenografts successfully resulted in functional sperm in the mouse host [222]. In a 2006 study, Turner et al. evaluated a similar xenografting procedure of stallion testis tissue in mice. Small pieces of testicular tissue were taken from normal stallions, cryptorchid stallions and stallions with ITD. The tissue was placed under the dorsal skin of immunocompromised and castrated mice. In normal testicular tissue, spermatogenesis resumed within the tissue [223-225]. The cryptorchid stallions also had evidence that spermatogenesis resumed. In stallions with ITD, the tissue did not resume normal spermatogenesis and rather underwent further degeneration suggesting that the testis itself is defective. The xenografts from stallions with ITD were also treated with exogenous and endogenous hormones which had no effect on the degenerating tissues [224,225]. This most likely proves that in stallions with ITD, hormone treatment is not likely to improve the condition.

An accurate diagnosis of TD begins with a precise breeding history of the stallion. The history should include the stallions past book sizes, seasonal pregnancy rates, and average numbers of heat cycles per pregnancy in each mare. A detailed history may also provide information regarding a possible cause for the TD. A recent history of trauma to the testis, an illness associated with fever, administration of anabolic steroids or administration of other likely damaging substances. In these cases, the onset of infertility is generally sudden and closely associated with the cause. The prognosis for future

fertility is generally better in cases where an inciting cause can be identified and removed, than for cases of true ITD. Stallions, especially older ones that present with no history of a known insult and declining fertility over time should be considered for ITD. ITD is typically considered to be a slowly progressive problem, however many stallions present for an alleged acute onset of subfertility/infertility.

Testicular hypoplasia (TH) is a congenital condition whereas TD is an acquired condition thus, a history of declining fertility and often decreased testicular size is critical for a diagnosis. It should be kept in mind that many animals with TH often are affected by degeneration as well [202,207]. Clinically and histologically, TD may be impossible to differentiate from TH. TD is strictly an acquired condition as opposed to TH, which can be congenital or acquired. These two terms are often mistakenly used interchangeably, so TH will be briefly described here.

TH is a fairly common pathologic entity of stallions. One study evaluating 1000 stallions found a 3% incidence in which it was associated with concurrent epididymal hypoplasia [67]. The difference between TH and TD is that TH is differentiated by incomplete gonadal development and TD or atrophy, involves gonadal regression after maturation is complete.

When a stallion reaches puberty, advanced development within the testes make them a sexually functional organ. Any event that impedes prepubertal development leading up to puberty can result in TH. TH is thought to be a consequence of primarily congenital abnormalities although it may be acquired [226], genetic and/or teratogenic. Some types of TH have a hereditary basis in some species, so genetically induced TH of horses is

possible [67,226]. The pathophysiology of TH may involve abnormal primordial germ-cell activity during fetal life, such as inadequate proliferation in the yolk sac, improper migration en route to the fetal gonad, or insufficient multiplication/excessive degeneration after arrival at the gonad; or it may involve postnatal disruption of the germinal epithelium [227]. A wide range of cytogenetic abnormalities, from translocations and mosaics to nondysjunctions causing polysomies of sex chromosomes, result in TH. The best known example of polysomy is the XXY karyotype of Kleinfelter's syndrome seen in bulls, dogs, boars, stallions and tricolor cats [207].

TH occurs in connection with cryptorchidism (both intersex and idiopathic forms) but also occurs as an unexplainable phenomenon of intrascrotal testes. Elevated testicular temperature may contribute to hypoplasia leading to the production of small testes when thermoregulation is impaired (e.g., with abdominal or inguinal cryptorchidism, inguinal herniation, congenitally short cremaster muscles or excessive intrascrotal fat in bulls) [226]. A disruption in thermoregulation can lead to TD and atrophy while TH may occur when such conditions as those above are present prior to the completion of testis growth and development. Some flocks of sheep have a high incidence of TH occurring concurrently with unilateral cryptorchidism, suggesting that the two conditions may be related [67]. In addition, the testes of male equine hybrids (mules or hinnys) are also typically hypoplastic.

In addition to genetic errors, it is possible that infections, intoxications, malnutrition, endocrinologic disturbances, irradiation or other factors may activate TH. An example of this type of TH would be small testes size in a young stallion retired from racing. In general, stallions with a racing career have smaller testes than stallions the same age not

involved in racing. This implies that vigorous training and/or possible drug administration negatively affect testis size [228].

TH can be mild to severe and can involve one or both testes. Resulting fertility is determined by the degree of hypoplasia [67,203] with clinical findings varying accordingly. Testicular size and consistency are two simple and noninvasive ways to evaluate the severity of TH. Hypoplastic testes are usually small but may occasionally be of normal size [67]. The texture of the affected testes vary from normal to soft with mild or moderate hypoplasia, to firm in severely affected testes. In severe cases, the testes become firm due to the large amount of stromal connective tissue present [203]. The testes of a suspect stallion should be compared with those of similarly aged stallions. TH can be unilateral or bilateral but the left testis tends to be larger than the right one during fetal development, with the size difference continuing into adulthood [63,229-231].

Prepubertal testes are quite small and can be mistakenly diagnosed as pathologically hypoplastic. Testicular growth in prepubertal/pubertal stallions usually increases at a fairly rapid and linear rate between 12 and 22 months of age, but testicular growth continues until at least 4-5 years of age [63,164,231]. Puberty may be delayed because of malnutrition or other factors; therefore, diagnosis of pathologic TH can be quite difficult before a stallion reaches 2-3 years of age.

A young postpubertal stallion with testes smaller than normal should be suspected of TH. TH is difficult to differentiate between TD when the duration of small testicular size is not known. Physical examination findings, testicular histologic findings and semen characteristics are very similar between the two pathologic states [67]. If an inguinal

herniation or some other congenital condition is present, there is a high likelihood that testicular development was impaired. An underdeveloped or small epididymis associated with a small testis provides indirect evidence for TH.

In mild cases of TH, most of the affected seminiferous tubules are undergoing active spermatogenesis progressing to the primary spermatocyte stage or beyond with some tubules remaining completely hypoplastic. Hypoplastic seminiferous tubules can be found scattered among numerous tubules that appear normal histologically. Stallions with a mild case of TH tend to have slightly small testes with a normal turgid to soft consistency that is hardly noticeable on physical examination. Stallions with severe TH are characterized by predominantly hypoplastic seminiferous tubules. These tubules are typically lined only by Sertoli cells, though some contain a small rim of non-dividing spermatogonia or spermatogonial precursors. The affected tubules have a thickened basement membrane that is infiltrated with hyaline connective tissue with the connective tissue also accumulating in the interstitial compartment. The testes are smaller than normal because the diameter of the seminiferous tubules is reduced. Although the testes may be soft at first, gradual replacement of parenchyma with connective tissue eventually makes the testes firm in texture [67,203]. Mild hypoplasia is difficult to distinguish from TD [207].

Affected stallions can have oligospermia leading to azoospermia depending on the severity of TH. Libido and semen volume are often normal. Ejaculated spermatozoa usually have a high incidence of morphologic defects and poor motility. Round spermatogenic cells and multinucleated germ cells may appear in the ejaculate as a result of incomplete cytoplasmic divisions during spermatogenesis [203].

There is no known treatment for stallions with TH, thus hypoplastic testes are predisposed to TD. Stallions with TH should not be used for breeding purposes as the condition is likely to be hereditary, and the stallions have reduced fertility. Unfortunately, the value of some stallions encourages owners to breed them anyways.

Azoospermia is often seen in TD within the first two weeks following a known insult. If the inciting factor is removed, it takes approximately 57 days for next spermatogenic cycle to produce viable spermatozoa. Upwards of 5 months may be required for recovery and return to function in cases of severe TD [200]. As mentioned previously, the spermatogonial stem cells and testicular somatic cells (Leydig cells and Sertoli cells) are fairly resistant thus they provide a population of cells that are capable of repopulating the testis following an insult.

In one study evaluating germ cell loss rate in stallions with ITD it was noted that stallions with mild cases of ITD may not have any obvious changes in testicular character and that ITD can be present before a clinically significant decrease in testicular size can be appreciated [221]. Semen quality should be monitored frequently as a gradual decline in quality (including a decline in total sperm numbers and/or declines in the percentages of motile and morphologically normal sperm) may be the only clinical sign early in the disease. Decreasing testicular size, palpable softening of the testicular parenchyma, decreasing sperm numbers, low daily sperm output per ml of testis, appearance of increasing numbers of immature round spermatogenic cells and/or multinucleate giant cells in the ejaculate and an overall decline in semen quality may become more apparent as the disease progresses [202,203,208,232]. Azoospermia can be found in advanced cases of ITD in addition, the epididymis may seem strangely large with respect to

testicular size because ITD doesn't affect it [213]. Some stallions with ITD present for an acute onset of subfertility/infertility when in reality the problem has been progressing over time but was not noticed. The testes may become overly firm in severe, end-stage ITD [5].

Thorough yearly examinations should be done on all breeding stallions with detailed records of total scrotal width, daily sperm output, and testicular volume. Meticulous record keeping will allow early detection of trends indicating ITD (e.g., decreasing testicular size/volume, declining semen quality, declining sperm numbers). If a stallion has a low daily sperm output for his testicular volume, he should be suspected of having ITD. The volume of a single testis can be calculated using the following formula [97]:

$$\text{Testis Volume} = \frac{4\pi}{3} \times \frac{\text{Length (cm)}}{2} \times \frac{\text{Width (cm)}}{2} \times \frac{\text{Height (cm)}}{2}$$

AND

$$\text{Total Testicular Volume} = \text{Left Testis Volume} + \text{Right Testis Volume}$$

Additionally, daily sperm output per ml of testis can be calculated by dividing the total number of sperm in the ejaculate by the total testicular volume [232]. TD is often indicated in stallions with low daily sperm output/ml of testis and a low percentage of morphologically normal sperm in the ejaculate [232].

ITD, TD and HP can all have large numbers of immature spermatogenic cells (round cells) and multinucleated giant cells in the ejaculate [202,203,208]. These cells can

sometimes be confused with white blood cells in an unstained semen sample. Different stages of spermatogenic cells appear in a single ejaculate and are thus varied in size compared to white blood cells. Spermatogenic cells can sometimes be found in the ejaculate of normal stallions but no other signs of abnormal spermatogenesis (small testicular size, poor semen quality, low sperm number, etc) should be present [233]. Plasma hormone levels are not a good predictor of mild to moderate TD as the levels can vary significantly in normal and subfertile stallions [221]. High plasma levels of FSH and LH with low plasma estradiol are often helpful in diagnosing of TD.

When incised, degenerate testes do not bulge and are dark brown [203]. Histologic evidence of TD includes cytoplasmic vacuolization, germinal-cell desquamation, decreased thickness of the seminiferous epithelium, decreased cross-sectional diameter of the seminiferous tubules, pyknosis of spermatocyte nuclei, intratubular giant cell formation, spermiostasis, mineralization of thickened tubular elements, diminished tubule size, fibrosis, and apparent interstitial cell hyperplasia [67,203,207]. There is loss of or reduced spermatogenesis and small tubules may be lined only with germinal cells that can be shed into the lumen. Hyaline thickening and occasional mineralization of the basement membrane may be wavy as a result of the collapse of the seminiferous tubules [67,203,206,207,234]. In more severe cases, large numbers of immature round spermatogenic cells may appear in the ejaculate, as described above. Germ cells decrease in number as TD progresses and in severe cases, fibrous tissue may become abundant and seminiferous tubules become almost devoid of spermatogenic cells. The testicular parenchyma may also become fibrotic and calcified [235]. Normal testes can have some focal areas of abnormal spermatogenesis. The percentage of the testicular parenchyma

that is affected and severity of the histological lesions should be considered before a diagnosis of TD is made.

A testicular biopsy will help provide evidence that can aid in the diagnosis of TD but the procedure is rarely performed in practice. A thorough history, complete physical examination and a reproductive examination often give a practitioner enough evidence to make a diagnosis of TD without taking a testicular biopsy. There is considerable debate regarding the significance of a single biopsy sample as a representative sample of the entire testis. Some researchers say that a wedge biopsy is preferred to obtain sufficient testicular parenchyma. This type of biopsy can result in considerable hemorrhage in addition to pressure-induced degeneration and necrosis [236]. Decreased sperm counts and formation of antisperm antibodies are among the reported complications in men [237]. An ultrasound examination should be used to evaluate the testes prior to taking a biopsy [238]. The ultrasonographic appearance of the parenchyma can help the practitioner choose a representative site for the biopsy. Several stallion studies have shown that testicular biopsies can be taken safely and with minimal permanent damage to the remaining testicular parenchyma [239,240]. The problem with the results of these studies is that they were performed on normal stallions and thus the risk to an already compromised testicle (e.g. degenerating testicle) is more difficult to determine. A case report on a stallion exhibiting signs of azoospermia determined via a testicular biopsy that the azoospermia was due to testicular degeneration and not obstructive purposes. This stallion already had one testis removed prior to this incident and his fertility status was already in jeopardy prior to the biopsy [206]. Practitioners must carefully weigh the

diagnostic benefits of obtaining a biopsy sample against the risk of damaging some portion of an already marginally functional testicular parenchyma.

There is no known, proven successful treatment for TD. If the cause of the degeneration is known (e.g., fever, toxin), successful treatment of or removal of the inciting cause should at least prevent further progression of the disease. If the degeneration is not severe and if the inciting cause is removed, the testis may at least partially, and sometimes fully, recover depending on the degree of damage sustained.

It has been recommended in cases of unilateral TD that the affected testis be removed because the damaged testicular tissue could possibly result in the production of antisperm antibodies [241,242] that might adversely affect sperm produced by the normal testis. In humans, autoimmune-related infertility is often associated with antisperm antibodies in women [209]. It is also thought that late descent of inguinal testes occurs in horses [243] and it could be hypothesized that late descent could present as TD. In addition, the remaining testis results in testicular hypertrophy thus increasing sperm numbers. Unilateral castration is of considerable debate since reports have stated that stallions with unilateral TD have acceptable fertility without removal of the affected testis [200].

GnRH therapy has proved successful in several studies for the treatment of infertility in stallions [244,245,245] but the results have not been duplicated in control studies [215,246,247]. Men with hypogonadotropic-hypogonadism have been successfully treated with GnRH therapy [248]. This condition has not been clearly documented in stallions. Therefore the use of GnRH implants or pulsatile administration of GnRH as a treatment for stallion infertility or ITD is debatable. This treatment may benefit the

stallion if therapy is attempted before the testis has reached a severe state of degeneration [249] but as Turner et al. discovered, testicular tissue from stallions with ITD is not responsive to hormone therapy [224].

A study by Brinsko et al. found improvements in several parameters of semen quality when a nutriceutical rich in docosahexaenoic acid (DHA) was fed to normal stallions [250]. The effect of this nutriceutical on stallions with infertility has yet to be determined.

Since the possible treatments for TD are of unknown value, stallion management should be monitored closely. Meticulous records should be kept on breeding stallions closely monitoring the number of progressively motile, morphologically normal sperm that the stallion is capable of producing on a regular basis. The stallion should then be used accordingly to prevent overuse. Stallions with marginal fertility should have limited breeding days with adequate rest between ejaculates to allow for maximum insemination doses. This rest period often helps boost sperm numbers and improves pregnancy rates in mares. Each ejaculate should be closely monitored to ensure that spermatozoal numbers are adequate for breeding. Sperm longevity and motility can often be improved with the use of an extender.

Stallions with TD should have their semen handled with extreme care and often mares should be inseminated immediately following collection. Not all semen from stallions with TD is suitable for cooled transport. The semen from stallions with moderate to severe TD is often not able to withstand the cooling and shipping process resulting in decreased pregnancy rates. Stallions with poor seminal characteristics are often best used

for onsite breeding or natural cover. Mares bred to stallions with TD should be intensely managed and bred close to ovulation to minimize sperm longevity. Intracytoplasmic sperm injection has also been evaluated in horses and could possibly benefit stallions with infertility [251].

Stallions with an identifiable cause of TD typically have the best chances of returning to a fully functioning status depending on the degree of damage. If a case of TD has an unknown cause then is should technically be considered ITD. Hormone levels and gene expression have not shown to be foolproof when attempting to detect early stages of ITD. A thorough history and annual breeding soundness examination will help detect small changes over time. Presently, there is no known successful treatment for ITD. If this disease is caught early, and breed registries permit, semen can be frozen in expectation of a steady decline in the stallion's fertility over time. Stallions diagnosed with ITD should be managed intensely to maximize fertility as the semen quality will continue to progressively decline. This disease can have a huge impact on the genetics of the equine industry thus requiring additional research to identify and diagnose early stages of ITD.

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CHAPTER III

A SURVEY OF TESTICULAR LESIONS IN STALLIONS

ABSTRACT

Very little is known or has been published about the incidence of testicular lesions in the general equine population. The aim of this survey was to assess the incidence of microscopic lesions in a random sample of pubertal stallions of different ages. Testicular samples from 65 adult stallions were fixed, processed and stained for microscopic evaluation. A board-certified pathologist evaluated the histological slides and assigned grades to the sample based on distribution and severity of any lesions. Organs and regions examined for lesions included seminiferous tubules, testicular interstitium, rete testis, epididymis or capsule. In the seminiferous tubules, 89% of the sampled stallions had evidence of tubular degeneration and 31% had evidence of tubular atrophy. Tubular dilation, intraepithelial cysts and intratubular giant syncytial cells characterized the degenerated seminiferous tubules. Intratubular granulomatous inflammation was present in 38% of the stallions. A malignant seminoma was identified in one stallion. Of the 65 stallions, 92% had significant tissue alterations presenting as interstitial edema, Leydig cell hypocellularity, perivascular lymphocytic inflammation and interstitial fibrosis. The majority of the lesions graded as minimal to mild; some were moderate or severe. No significant tissue alterations existed in the rete testis, epididymis or capsule. This survey indicates that the incidence of testicular lesions in the general equine population is high

and that their severity is minimal to mild. Knowledge gained from this study will help to guide testicular diagnostic testing procedures in stallions in the future.

Keywords: Stallion, Survey, Testis

INTRODUCTION

The process of spermatogenesis is extensive, delicate and can easily be disrupted by a variety of extrinsic and intrinsic factors [1]. The germinal epithelium is relatively susceptible to injury; however, due to the resilient nature of the stem-cell spermatogonia, Sertoli cells and Leydig cells, the re-establishment of normal spermatogenic function is possible in some cases [2].

Equine testicular tumors are uncommon and are rarely reported most likely due to the practice of castrating stallions at young age, which makes it difficult to access the true incidence of pathologic conditions [3,4]. Testicular neoplasia is rare and can be classified as primary, which are the most common tumors, and secondary [3-5]. Primary testicular neoplasms are classified based on their origin into two categories 1) germinal or 2) somatic (nongerminal). They can then be further classified based on their histologic features with germinal neoplasms originating from the germ cells of the seminiferous epithelium. Germinal neoplasms comprise a majority of testicular tumors [6] and include the seminoma, teratoma, teratocarcinoma and embryonal carcinoma. Somatic neoplasms include Leydig cell tumors, Sertoli cell tumors, and other nonparenchymal neoplasms such as lipomas, fibromas and leiomyomas [7]. Stallions castrated at an older age may have small neoplasms that are overlooked and therefore under-reported.

Multifactorial diseases such as testicular hypoplasia (TH) and testicular degeneration (TD) are also of considerable importance when evaluating equine testicular pathology. TH occurs before gonadal development is complete, whereas TD involves gonadal regression after maturation is complete. TH is fairly common in stallions and often occurs in connection with cryptorchidism [5]. It is thought to be a consequence of primarily congenital abnormalities but other causes include genetic, teratogenic and acquired [8]. TD is a common cause of acquired, progressive infertility and subfertility in stallions that often leads to sterility and contributes significantly as a loss to the equine industry [5,9-17]. TD can manifest in two ways: 1) acutely and secondary to a known insult on the testis or 2) one in which no underlying cause can be found. Known causes of TD include, high environmental temperature, fever, orchitis, periorchitis, hydrocele, scrotal hemorrhage, scrotal edema, scrotal dermatitis, improper scrotal descent, systemic/local infections, injury to essential vasculature (torsion), hormonal disturbances, radiation, malnutrition, ingestion of toxic plants, neoplasia, efferent/epididymal duct obstruction, production of antisperm antibodies and many more [5,6,10,12,13,17-21]. TD can often be reversible depending on identification and removal of the insult and the length and severity involved. TD in which no underlying insult is identified is referred to as idiopathic testicular degeneration (ITD) [11,13,16]. ITD is often associated with aging and can be found in middle-older age stallions but younger stallions can be affected too [13]. Stallions with ITD often show signs of decreased fertility, but the condition is progressive in nature and often leads to sterility. ITD is not responsive to hormone therapy and is most likely due to a defect in the testis itself [22-25].

Routine evaluation of the breeding stallion typically involves a physical examination, semen analysis, bacteriological cultures, ultrasonography and hormonal assays [26]. Testicular biopsies are often only performed when less invasive techniques are in doubt for diagnosis [27]. A thorough history, complete physical examination and a reproductive examination often give practitioners enough evidence to make a diagnosis without taking a testicular biopsy. Because of its perceived invasiveness, evaluations of testicular biopsies in the stallion are not routinely performed as a common procedure, thus limiting the use of biopsy as a diagnostic tool. Several studies have shown that biopsy and fine needle aspiration produces minimal long term effects on fertility in normal stallions [27-30]. Similar studies have yet to be performed in stallions that are already compromised.

Very little is known or has been published about the incidence of testicular lesions in the general equine population. Further complicating the matter, the field of equine medicine has yet to characterize testicular lesions in stallions which can be attributed to the normal aging process. To assess the incidence of microscopic testicular lesions in stallions, we surveyed a random population of pubertal horses. We hypothesize that the incidence of testicular lesions in the general equine population is low.

MATERIALS AND METHODS

Animals, samples and tissue handling

Testes were obtained from 65 pubertal stallions slaughtered for food consumption at an abattoir in Fort Worth, Texas between the months of March and June in 2003. Workers at the slaughter house severed the testes at the level of the spermatic chord, pampiniform

plexis and cremaster muscle. Testes were grossly examined for abnormalities and noted if present. Testes were processed for fixation on site to prevent autolysis. Because the testicles were too large to fix whole, they were cut into sections prior to fixation. Testes were cut axially and a representative, mid-testicular section was removed parallel to the cut. The sections were then immediately placed into Modified Davidson's solution (30% v/v of a 40% v/v formaldehyde solution, 15% ethanol v/v, 5% v/v, glacial acetic acid and 50% distilled water) and fixed at least 24 hours at room temperature.

Histology

After fixation, the testes were washed in phosphate buffered saline three times prior to being processed through graded alcohols and xylene routinely, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin for microscopic evaluation.

Evaluation of histological sections

The slides were examined by a board-certified pathologist at the Oklahoma Animal Disease Diagnostic Laboratory. The tissues were evaluated according to lesion location (seminiferous tubules, interstitium, rete testis, capsule, and epididymis) and severity (scale of 0 to 4 with 0 being normal and 4 being severe). The severity was characterized based on the percent of tissue affected: minimal was defined as <10% affected; mild was >10% but <20%; moderate was >20% but <30%; and severe was >50% of the tissue affected.

RESULTS

The results are summarized in the TABLE 1. A majority of the testicular tissues examined had microscopic lesions present (Table 1). The testicular lesions varied from minimal to mild with some lesions grading moderate and few lesions were occasionally severe.

Normal stallion testis histopathology can be seen in Figure 2. The seminiferous tubules are round, contain multiple concentric layers of developing germinal cells and are actively undergoing spermatogenesis. The interstitium contains abundant numbers of Leydig cells.

Seminiferous Tubules

Of the 65 stallions examined, 89% had evidence of seminiferous tubule degeneration with 59% of those grading minimal in severity. Figure 3 characterizes diffuse seminiferous tubule degeneration. The germinal layers in the seminiferous tubules are mildly hypocellular, disorganized, and are lined by a collection of Sertoli cells and epithelial cells, with clear, cystic-like spaces or "intraepithelial cysts".

Intratubular inflammation was present in 38% of stallions. Twenty-one of twenty-five stallions graded minimal in severity.

Seminiferous tubule atrophy affected 20/65 stallions with 30% of the stallions grading moderate in severity. Figure 4 is an example of diffuse seminiferous tubule atrophy. The seminiferous tubules are oblong to curved with a decreased diameter. Some tubules are

lined with only Sertoli cells while some are completely devoid of germinal cells. The interstitium is moderately fibrotic, devoid of Leydig cells and minimally inflamed.

At initial gross examination and fixation, a gross abnormality was identified in the testis of one stallion (Figure 5). On microscopic examination, this lesion was identified as a malignant seminoma (Figure 6). Within a scant fibrovascular stroma, sheets of highly pleomorphic and infiltrative neoplastic round cells have obliterated the testicular architecture. Cells have moderately abundant eosinophilic cytoplasm, with distinct borders. The hyperchromatic nuclei are round to oval, with marked anisokaryosis and prominent, often multiple nucleoli; multinucleated giant tumor cells are frequent. Mitoses vary from 0 to 5 per high power field with no vascular invasion detected.

Interstitium

Of the 65 stallions, 92% had recognizable tissue alterations in the testicular interstitium. These consisted of interstitial edema (49/65), Leydig cell hypocellularity (42/65), perivascular lymphocytic inflammation (33/65), and interstitial fibrosis (11/65).

Interstitial edema was evident in 75% of stallions (49/65). Forty-six of the stallions were minimally to mildly affected. The interstitial tissues are moderately expanded with loosely arranged connective tissue and lymphangiectasia (Figure 7).

Leydig cell hypocellularity was identified in 42/65 stallions with 11 minimally affected, 15 mildly affected, 11 moderately affected and 5 severely affected. The basement membranes of the seminiferous tubules are wavy and are lined by a collection of Sertoli cells and epithelial cells, with clear, cystic-like spaces or “intraepithelial cysts”.

Spermatogenesis is decreased to absent in some tubules. The interstitial tissue is severely depleted of Leydig cells and has a mild non-suppurative inflammation (Figure 8).

Nearly half of the stallions had evidence of interstitial inflammation (33/65) grading minimal to mild in severity. A mild perivascular collection of lymphocytes can be seen expanding the peritubular interstitium (Figure 9). The seminiferous tubules surrounded by inflammation are not undergoing spermatogenesis.

Only 17% of stallions showed evidence of interstitial fibrosis. The seminiferous tubules are indented and clustered together. There is a loss of Leydig cells in the interstitium which has been completely replaced by marked fibrosis (Figure 10).

Tissue alterations in the capsule were focal and minimal, and no tissue alterations existed in the rete testis or epididymis.

A Fisher's exact test was used to compare the lesion locations that were histologically graded to determine if significances exist among them. It was determined that seminiferous tubule degeneration was statistically significant when compared to intratubular inflammation and interstitial inflammation ($p<0.05$). Leydig cell hypocellularity was also statistically significant when compared to interstitial edema ($p<0.05$).

TABLE 1 (n = 65 stallions)

	Testicular Lesions	Number of Stallions with Lesions	Degree of Severity			
			Minimal	Mild	Moderate	Severe
Seminiferous Tubules	Tubular Degeneration	58/65	34/58	9/58	15/58	0/58
	Intratubular Inflammation	25/65	21/25	4/25	0/25	0/25
	Tubular Atrophy	20/65	11/20	3/20	6/20	0/20
	Neoplasia	1/65	N/A	N/A	N/A	N/A
Interstitium	Interstitial Edema	49/65	20/49	26/49	3/49	0/49
	Leydig Cell Hypocellularity	42/65	11/42	15/42	11/42	5/42
	Interstitial Inflammation	33/65	23/33	10/33	0/33	0/33
	Interstitial Fibrosis	11/65	1/11	9/11	1/11	0/11
Rete Testis		0	0	0	0	0
Capsule	Capsular Inflammation	7/65	7/7	0/7	0/7	0/7
Epididymis		0	0	0	0	0

DISCUSSION

This study suggests that the incidence of microscopic testicular lesions in stallions is high and that the majority of the testicular lesions have a minimal to mild degree of severity. While this survey implies that the incidence of microscopic testicular lesions in the general equine population may be high, the findings may not be representative of the general breeding stallion population nor each individual stallion's reproductive performance and semen quality parameters. The testicular tissues evaluated were obtained from healthy stallions of undetermined age that were slaughtered for food

consumption. At the time of the sample collection, the reason(s) for which these horses were removed from the general breeding population was unknown. A representative sample population of normal breeding stallions would be difficult to acquire due to the perceived invasive nature of biopsy; therefore the equine population subjected to this survey is likely the best available sample.

Intratubular inflammation and interstitial inflammation were present in 25/65 and 33/65 stallions, respectively. Autoimmune orchitis is a cause of inflammation in animals and occurs when there is a disruption in the blood-testis barrier. Spermatozoa get outside of the protected areas and are recognized by the immune system as foreign. The rete testis and efferent ducts are typically the starting sites for the process which begins with a granulomatous reaction [31]. A major disruption in the blood-testis barrier has also been identified in human males with the formation of antispermatozoan antibodies. Infections, trauma and neoplasia that damage the testis can result in antibody formation. Stallions experiencing trauma to the testis with resulting low sperm cell viability have been found to have antispermatozoan antibodies in their seminal fluid or serum [31]. Fisher's statistical analyses confirmed a statistically significant association between tubular degeneration and intratubular inflammation and also between tubular degeneration and interstitial inflammation. While microscopic recognition of these two sets of parameters in conjunction is commonplace, this data set confirms the association. Further, future exploration of clinical interventions with anti-inflammatory drugs and their effect upon restoration of testicular architecture and function could be a warranted future study.

Stallions typically have a multifocal, mild, subacute intertubular inflammation with no gross lesions evident [31]. In stallions, mild interstitial orchitis is common with interstitial lymphocytic foci, often perivascular, occurring in areas of tubule degeneration and vasculitis [2,31]. Similar lesions may be part of a generalized vascular involvement in equine viral arteritis [2]. Focal interstitial orchitis is commonly found in human testes removed at autopsy and also from the testes of prostate cancer patients. The cause of inflammation in these particular cases is still unknown. Atrophy of the germinal epithelium is often associated with severe interstitial orchitis and if bilateral may result in infertility [32].

Intratubular orchitis most likely arises from an ascending infection of the urethra, urinary bladder, ductus deferens and epididymis [2,31]. This inflammation typically begins in the seminiferous tubules but spreads to the interstitium, thus leading to granuloma formation when spermatozoa breach the tubule border. The seminiferous tubule outline is often preserved in the affected area. The seminiferous epithelium is destroyed and replaced by abundant macrophages and multinucleated giant cells that encircle neutrophils and debris [2].

The stallions in this study were not cultured for bacteria nor were any additional laboratory tests performed. A bacterial or other infectious etiology did not appear to be the cause of this inflammation however, without a detailed history of each stallion, we are unsure of their fertility status prior to slaughter.

Interstitial edema was noted in 49/65 stallions with a majority of the lesions being minimal to mild in nature (20/49 and 26/49, respectively). This finding is not described

in the literature and could possibly be subjective interpretation by the pathologist, or even possible disruption in the testicular tissue due to transportation and stress. The minimal to mild nature of this change prevented attribution of specific causation, though additional study pathologists could eliminate the bias of a single evaluator, and evaluation of a subset of non-abbatoir horses might control for the possibility of transportation or stress contributions. Fisher's statistical analyses detected a statistically significant association between interstitial edema and Leydig cell hypocellularity ($p<0.05$). Both of these parameters are poorly described in the literature thus this comparison is probably the first to draw this conclusion. The significance of this relationship deserves further study.

Testicular neoplasia in horses is fairly uncommon and is rarely reported. The true incidence in the general equine population is unknown due to the practice of castrating stallions at a young age [3]. This study identified a malignant seminoma in one stallion of unknown age (Figure 5 & 6). Seminomas are the most common testicular neoplasm in the aged stallion with teratomas being more commonly found young stallions [2,31,33,34]. Seminomas are more prevalent in cryptorchid testes, commonly involving the undescended testis but no association between cryptorchidism and seminoma occurrence has been proven in the horse [33,35]. Microscopically, seminomas have an intratubular or diffuse arrangement of large, polyhedral, discretely demarcated cells with a large nucleus, variable nuclear size, and very little cytoplasm. Giant cells, with either single or multiple nuclei and lymphoid nodules are sometimes present. Seminomas are seldom malignant but often locally invasive [2,31].

The variation in Leydig cell density in testicular tissue, with and without evidence of concurrent testicular degeneration, deserves further study. This finding did not appear to correlate with the season of the year (data not shown). In horses, an increase in Leydig cells is typically seen with an increase in age. This is unlike rats, who experience negligible change, and humans, who experience a decline in Leydig cell numbers. The Leydig cell population size, like that of germ cells or Sertoli cells, cycles yearly in the horse [17-19] however, the size of individual Leydig cells does not differ with season of the year [17]. This lack of seasonal influence on the size of individual Leydig cells in stallions may be contributed to their continued production of spermatozoa throughout the year [36,37]. The samples in this study were taken from March-June and should have indicated an increased number of Leydig cells. A study by Nagata et al. [38] showed that the administration of a common anabolic steroid, 19-nortestosterone, had serious effects on the interstitial compartment with a severe depletion of the number of Leydig cells and advanced atrophy in the cells that remained. It is highly unlikely that 42/65 stallions in this study were under the influence of steroids at the time of slaughter. It is also not known at this point whether Leydig cell hypocellularity seen in this study has an affect on the fertility of stallions but to our knowledge, this is the first report to describe such findings in horses.

In conclusion, this survey suggests that the incidence of microscopic testicular lesions in the general equine population may be high and that their severity is minimal to mild. Such findings may or may not be representative of the normal breeding stallion population in general and may not correlate with breeding soundness and semen quality parameters. Although a majority of the stallions in this study had some minimal to mild

form of pathology present, it is possible that these mild changes have no impact on fertility status. Due to the invasive nature of obtaining samples from normal stallions, the lesions described in this study are from the best available equine population. Taken together, this study provides a revised basis for clinical decision making regarding stallion fertility.

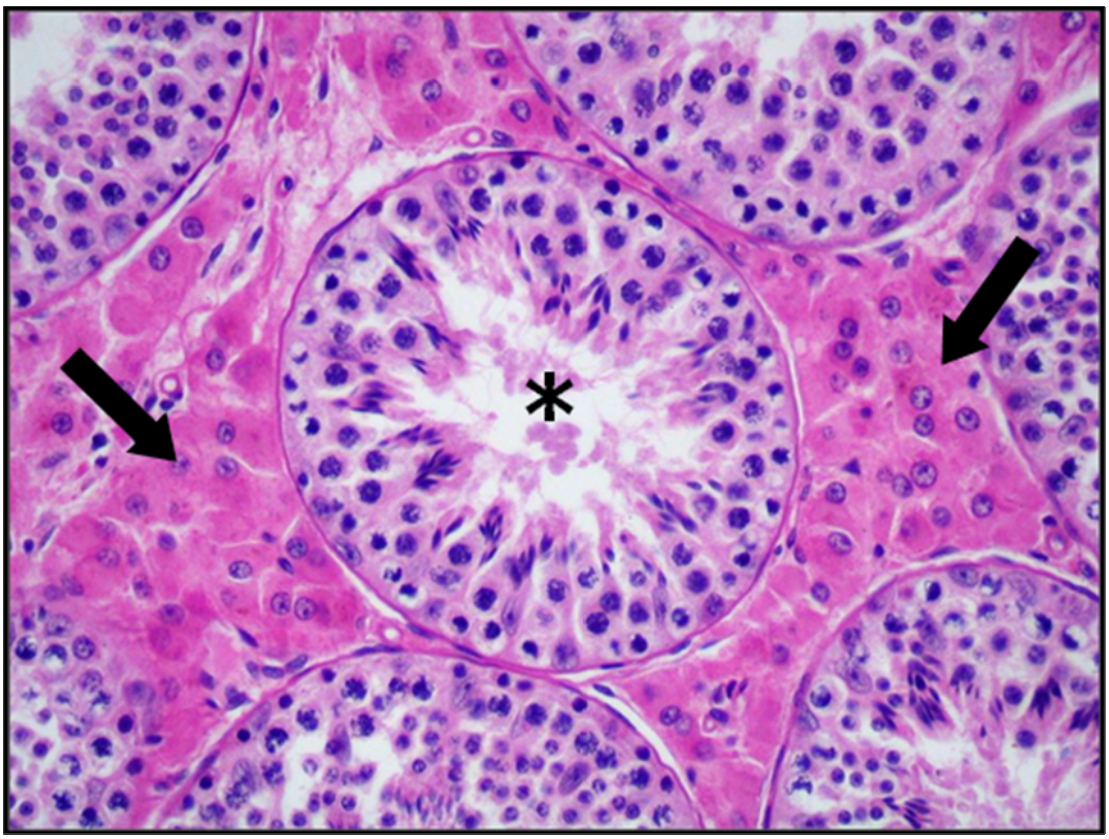

Figure 2 Normal Seminiferous Tubule and Interstitial Tissue

Note the normal seminiferous tubule (asterisk) and abundance of Leydig cells in the testicular interstitium (arrows).

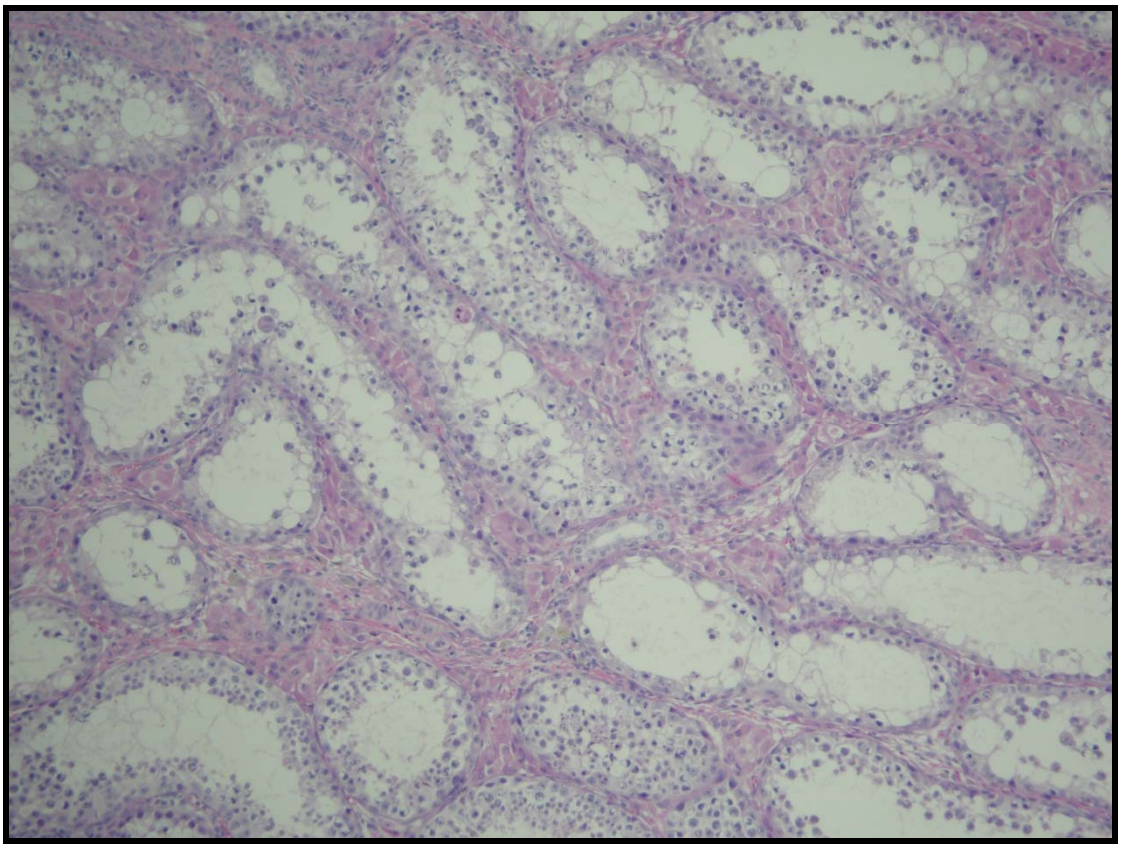

Figure 3 Diffuse Seminiferous Tubule Degeneration

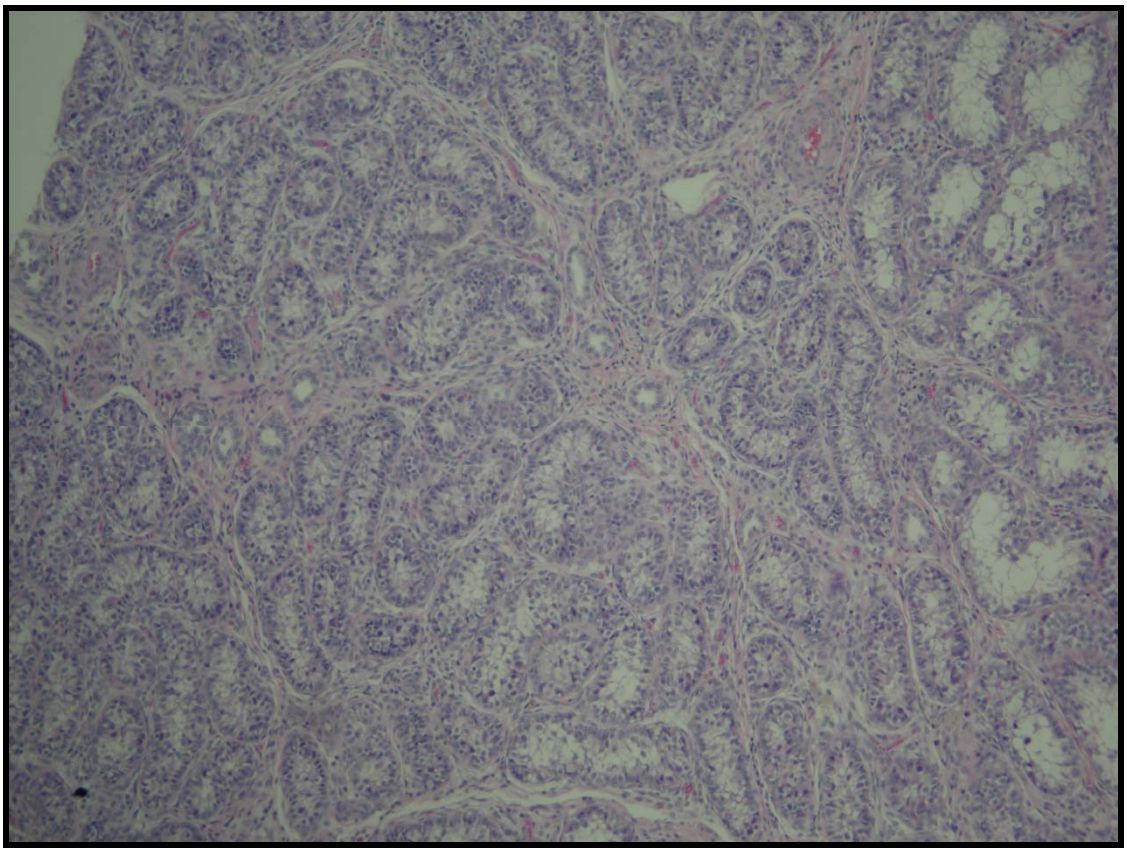

Figure 4 Diffuse Seminiferous Tubule Atrophy

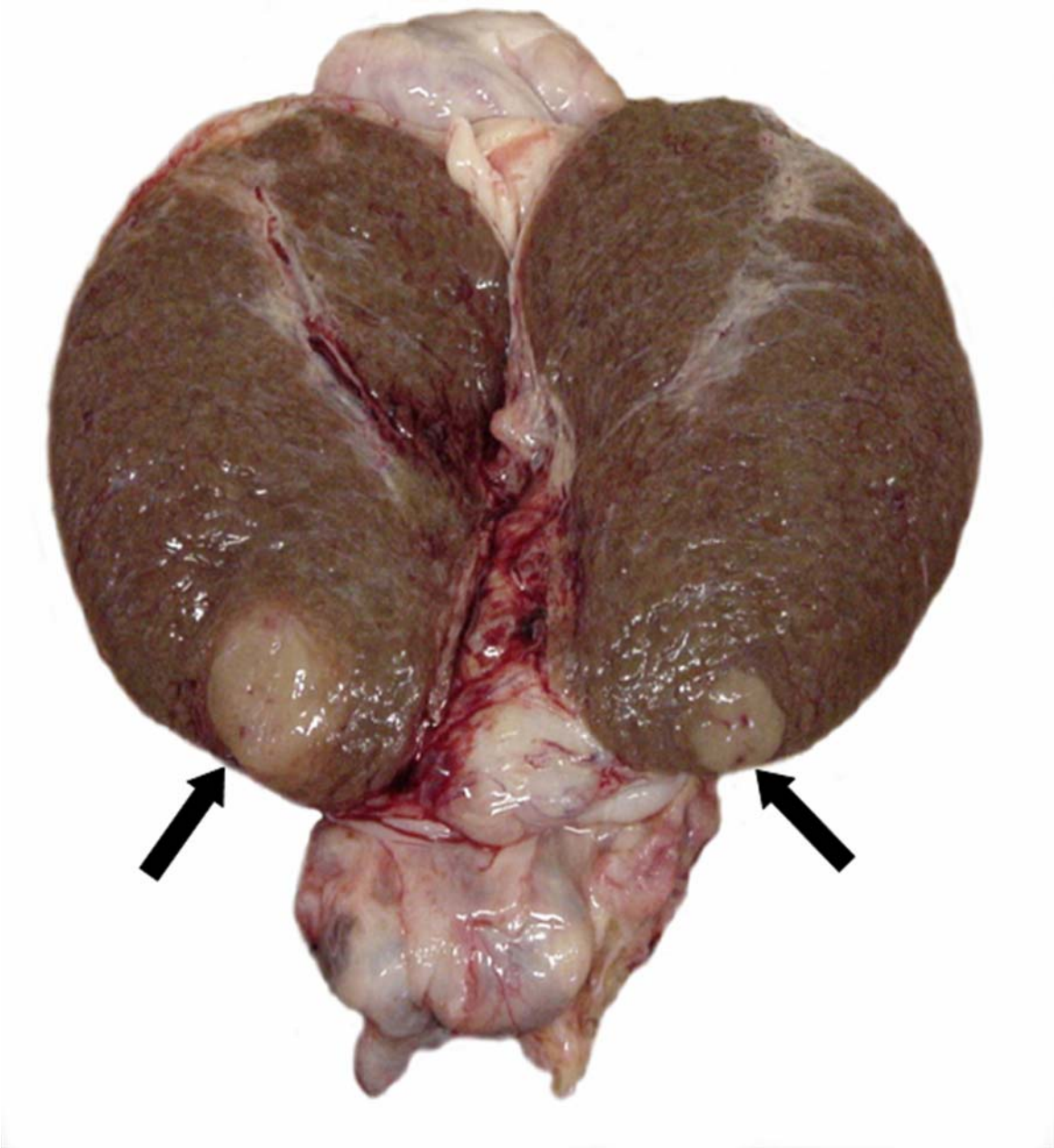

Figure 5 Gross Lesion of Testicular Seminoma

Lesion identified in the caudal pole of the testis on gross examination. Lesion was pale yellow, firm and bulged on cut surface.

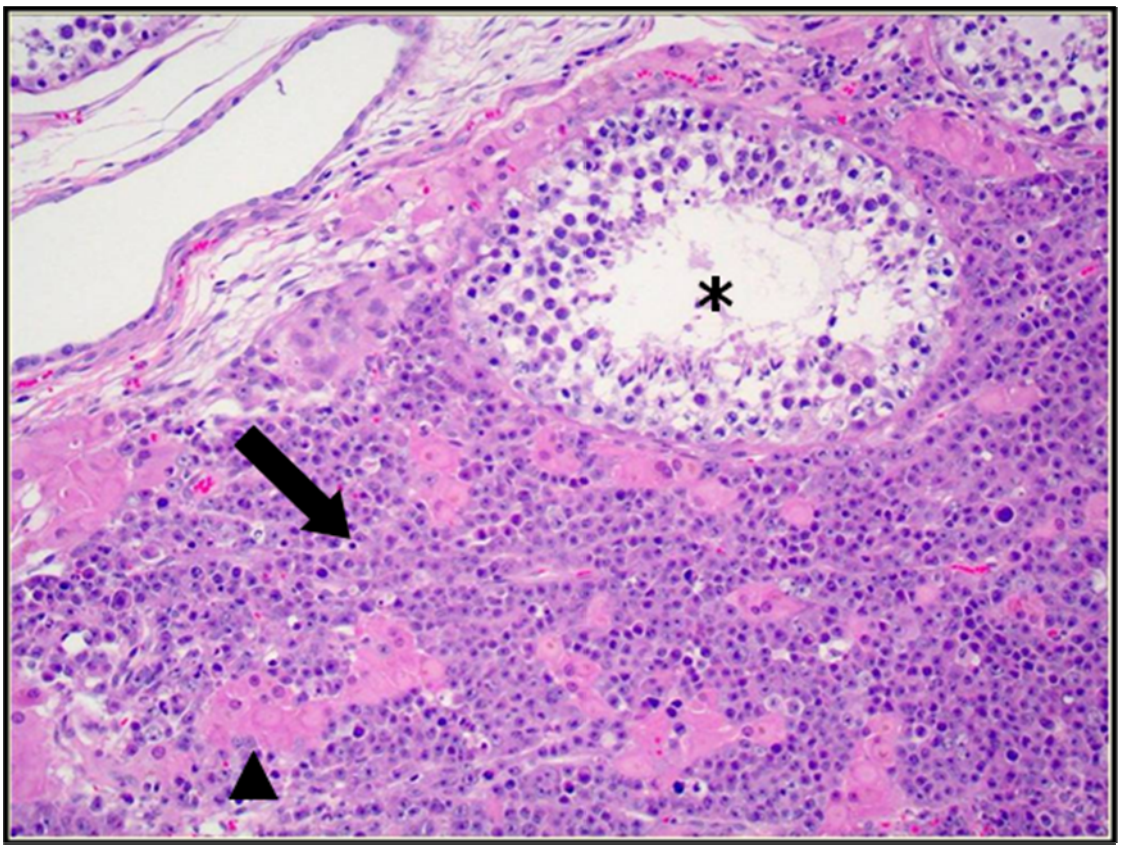

Figure 6 *Malignant Testicular Seminoma*

Malignant seminoma (arrow), seminiferous tubule (asterisk) and cluster of Leydig cells (arrowhead).

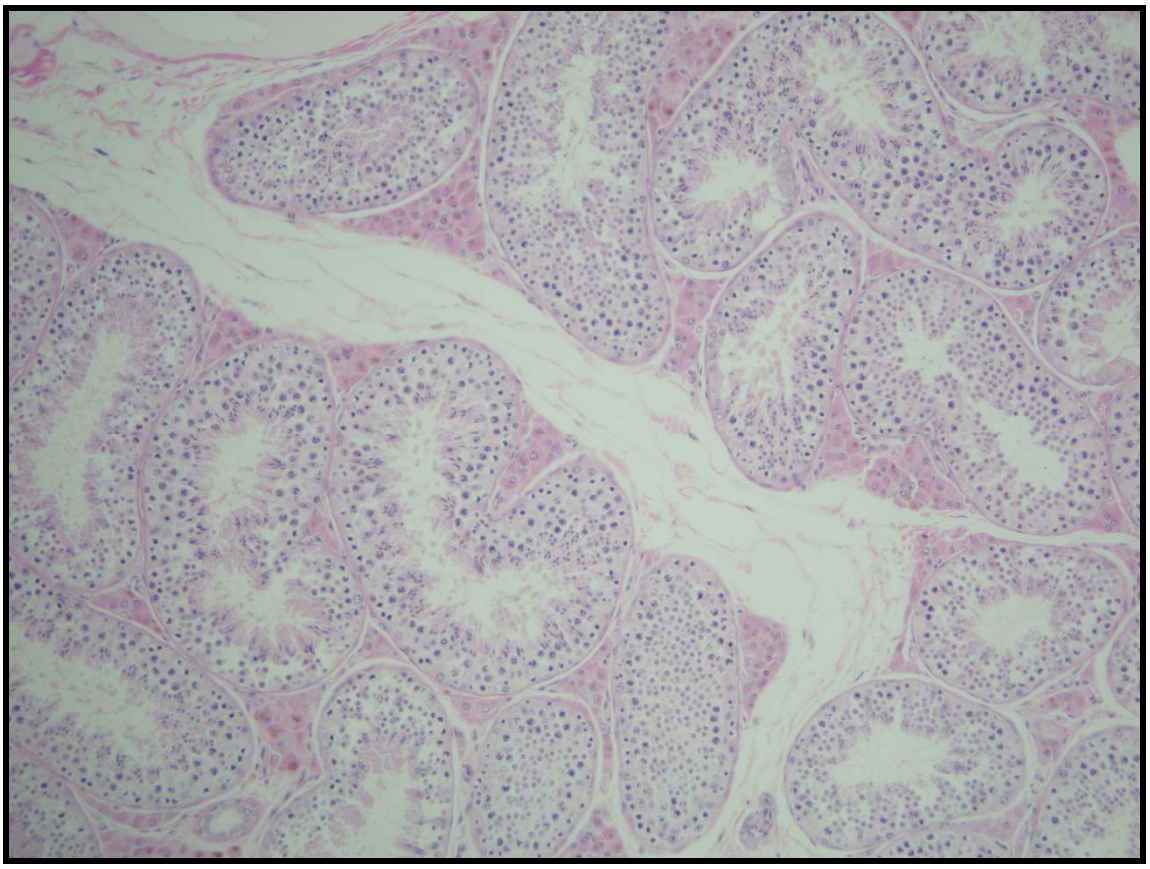

Figure 7 *Diffuse Interstitial Edema*

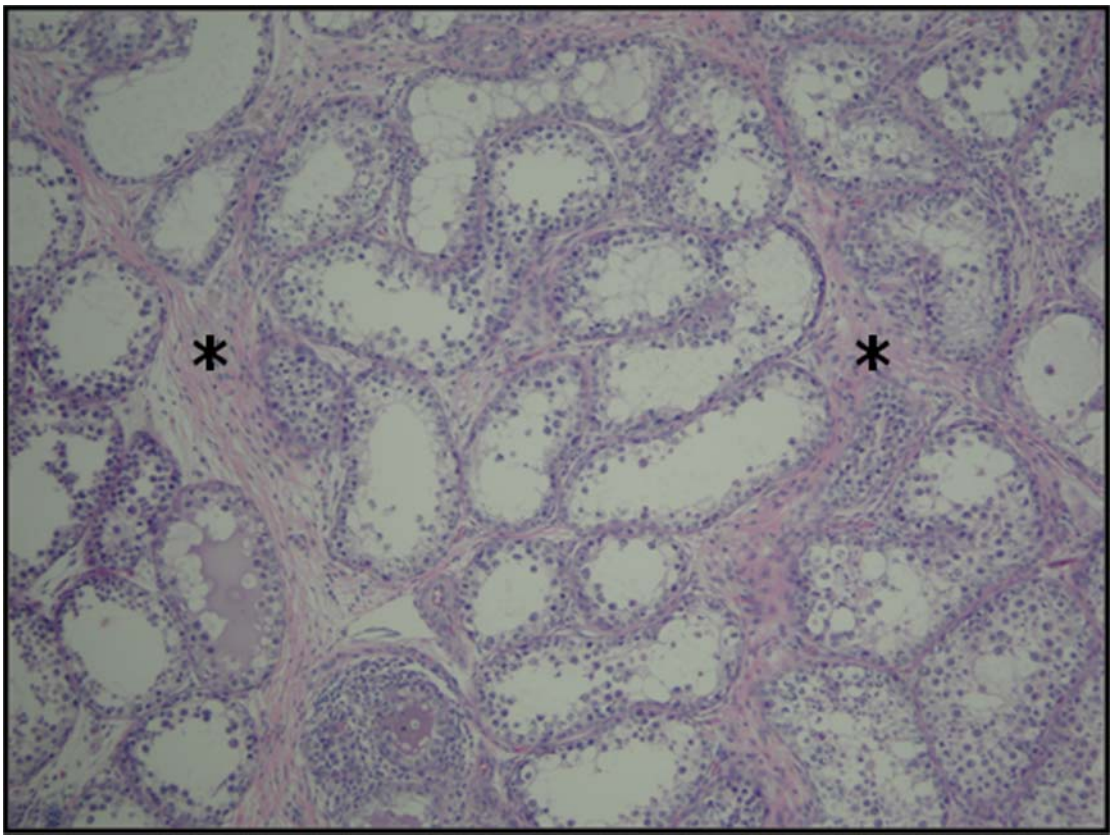

Figure 8 *Leydig Cell Hypocellularity*

Lack of Leydig cells in the interstitium (asterisks).

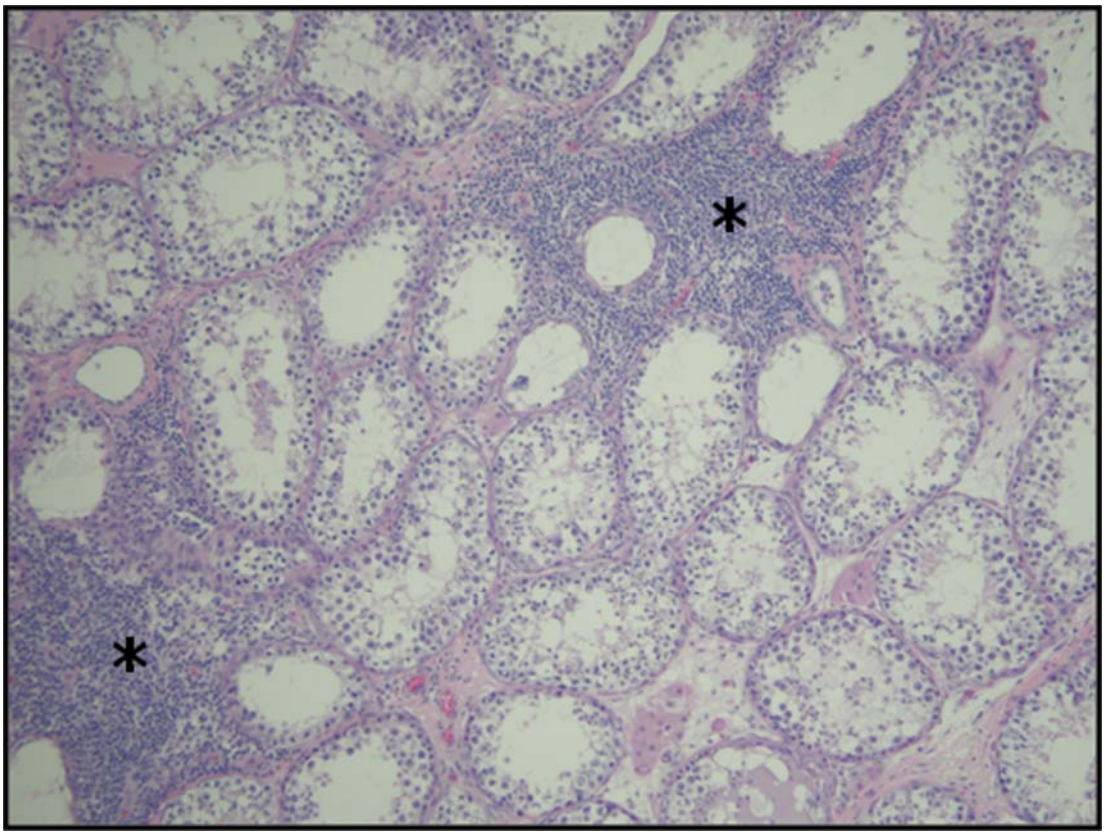

Figure 9 *Interstitial Inflammation*

Abundance of inflammatory cells in the interstitium (asterisks).

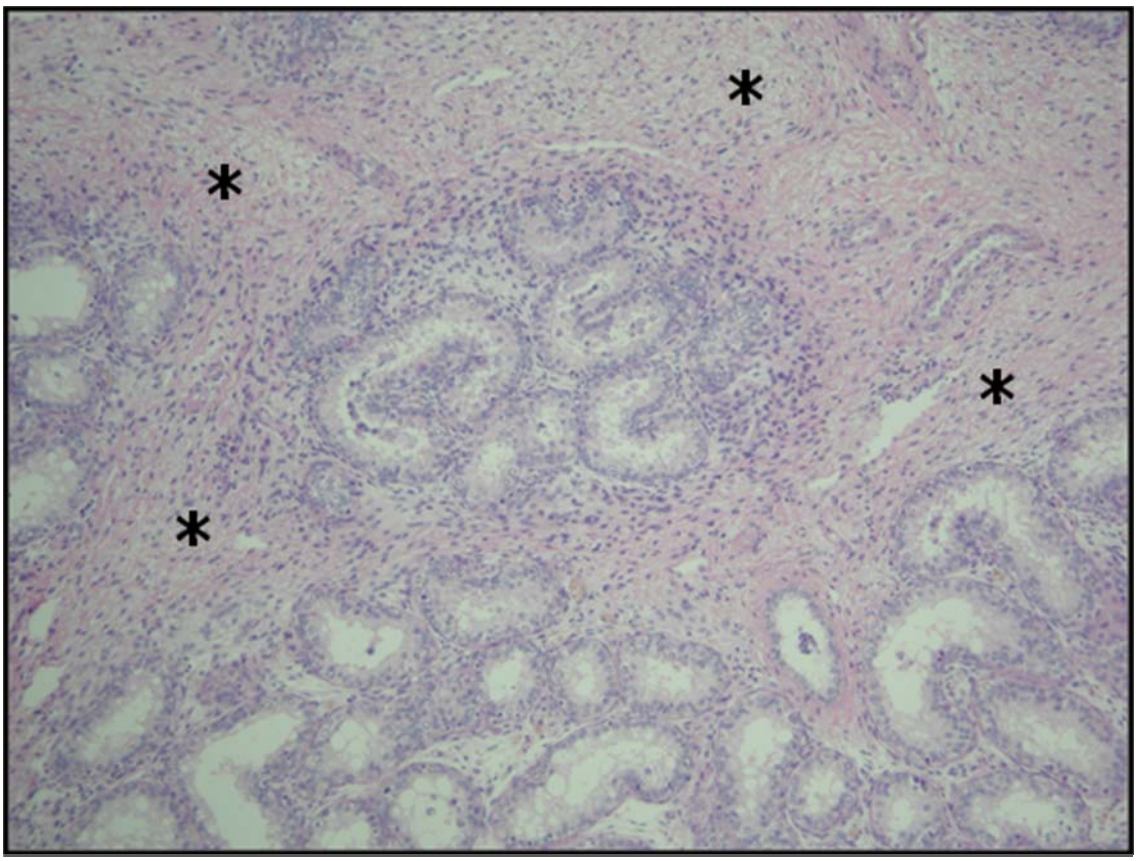

Figure 10 Interstitial Fibrosis

Severe interstitial fibrosis and lack of Leydig cells (asterisks).

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CHAPTER IV

CONCLUSION

The male reproductive system is extremely complex as evidenced here by the literature review provided. Many factors are capable of disrupting the delicate process of spermatogenesis thus resulting in a stallion incapable of impregnating mares. A thorough understanding of this complex system is mandatory when attempting to determine a cause.

The study provided here sampled the best available equine population to access testicular lesions that were present. Although a majority of the stallions in this study had some minimal to mild form of pathology present, it is possible that these mild changes have no impact on fertility status. This study provides a starting point for mapping the histopathologic changes associated with aging in the stallion.

This study has also served as a platform for several other projects. The first is a comparative fixative study for testicular and endometrial biopsies in the horse and the second is a study utilizing ultrasound as an early detection method for stallions with testicular degeneration.

The results of these studies will provide more answers regarding the complex events leading to infertility in the stallion. The future of equine genetics rests with those researchers attempting to further understand infertility in the stallion.

VITA

SHARLA MAE BIRCH

Candidate for the Degree of

Master of Science

Thesis: A SURVEY OF TESTICULAR LESIONS IN STALLIONS

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Data: Born in Sidney, Montana, on November 27, 1978.

Education: Graduated from Fairview High School, Fairview, Montana in May 1997; received a Bachelor of Science degree in Animal Science from Montana State University, Bozeman, Montana in May 2001; received a Doctor of Veterinary Medicine degree from Oklahoma State University Center for Veterinary Health Sciences, Stillwater, Oklahoma in May 2008. Completed the requirements for the Master of Science degree with a major in Veterinary Biomedical Sciences at Oklahoma State University in July, 2008.

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Title of Study: A SURVEY OF TESTICULAR LESIONS IN STALLIONS

Pages in Study: 118 Candidate for the Degree of Master of Science

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Findings and Conclusions:
Very little is known or has been published about the incidence of testicular lesions in the general equine population. The aim of this survey was to assess the incidence of microscopic lesions in a random sample of pubertal stallions of different ages. Testicular tissue from 65 adult stallions were fixed, processed and stained for microscopic evaluation. A board-certified pathologist evaluated the histological slides and assigned grades to the tissue based on distribution and severity of any present lesions. Tissue alterations were grouped by their location in seminiferous tubules, testicular interstitium, rete testis, epididymis or capsule. In the seminiferous tubules, 89% of the sampled stallions had evidence of tubular degeneration and 31% had evidence of tubular atrophy. Tubular dilation, intraepithelial cysts and intratubular giant syncytial cells characterized the degenerated seminiferous tubules. Intratubular granulomatous inflammation was present in 38% of the stallions. A malignant seminoma was identified in one stallion. Of the 65 stallions, 92% had significant tissue alterations presenting as interstitial edema, Leydig cell hypocellularity, perivascular lymphocytic inflammation and interstitial fibrosis. The majority of the lesions graded as minimal to mild; some were moderate or severe. No significant tissue alterations existed in the rete testis, epididymis or capsule.

Keywords: Stallion, Survey, Testis

ADVISER'S APPROVAL: Dr. Timothy Snider

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