Part 2: Male Reproductive System

Normal Physiology and Structure

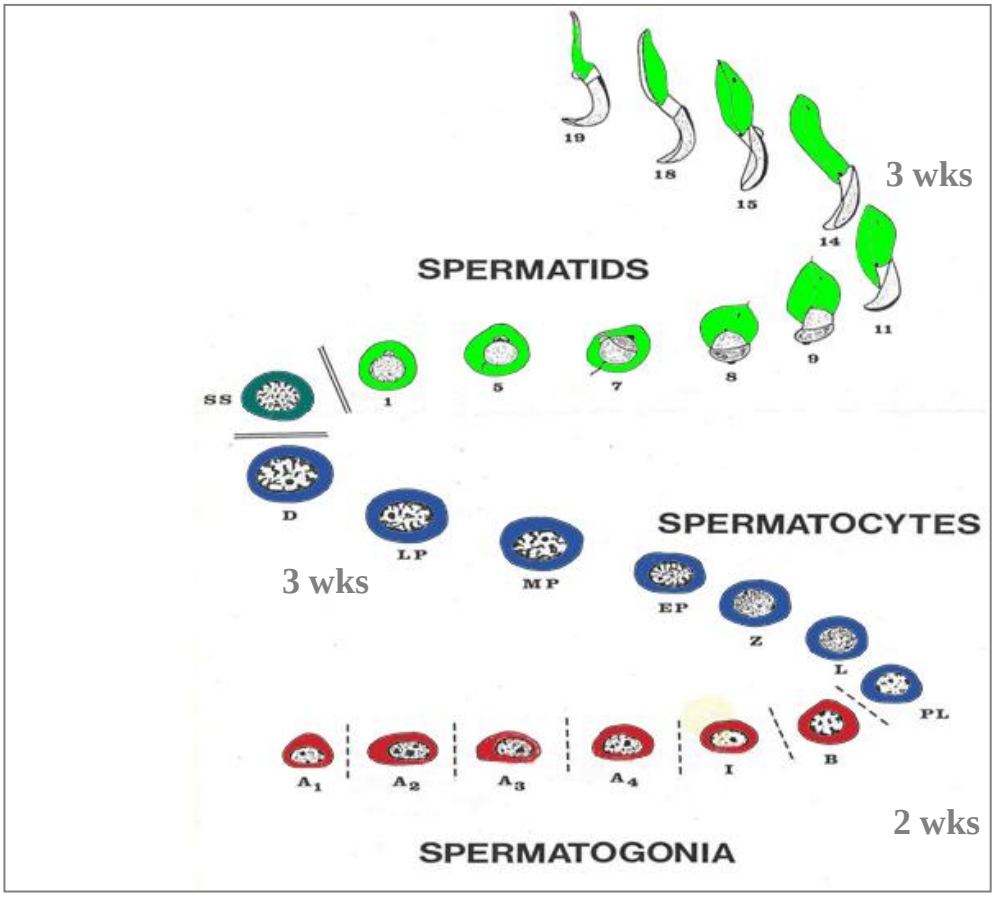
Testis

Function, physiology and regulation

The testis has two major functions: 1) producing sperm from stem cell spermatogonia (spermatogenesis) and 2) producing androgens, to maintain and regulate androgen mediated functions throughout the body.

Spermatogenesis

Spermatogenesis occurs in the seminiferous tubules, of which there are 10-20 in each rat testis. Spermatogenesis is the process whereby primitive, diploid, stem cell spermatogonia give rise to highly differentiated, haploid spermatozoa (sperm).



The process comprises a series of mitotic divisions of the spermatogonia, the final one of which gives rise to the spermatocyte. The spermatocyte is the cell which undergoes the long process of meiosis beginning with duplication of its DNA during preleptotene, pairing and condensing of the chromosomes during pachytene and finally culminating in two reductive divisions to produce the haploid spermatid. The spermatid begins life as a simple round cell but rapidly undergoes a series of complex morphological changes. The nuclear DNA becomes highly condensed and elongated into a head region which is covered by a glycoprotein acrosome coat while the cytoplasm becomes a whip-like tail enclosing a flagellum and tightly-packed mitochondria. The sequential morphological steps in the differentiation of the spermatid (19 steps of spermiogenesis) provide the basis for the identification of the stages of the spermatogenic cycle in the rat.

In a cross section of a seminiferous tubule, the germ cells are arranged in discrete layers. Spermatogonia lie on the basal lamina, spermatocytes are arranged above them and then one or two layers of spermatids above them. In any given normal tubule, four generations of cells develop simultaneously and in precise synchrony with each other. As each generation develops, it moves up through the epithelium, continuously supported by Sertoli cells, until the fully formed sperm are released into the tubular lumen (spermiation). The synchrony of the development between the 4 generations of cells is such that each successive stage of development of the spermatogonium is found with its characteristic spermatocyte and spermatids.

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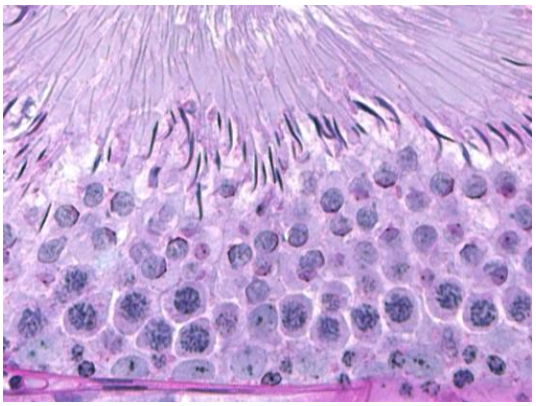

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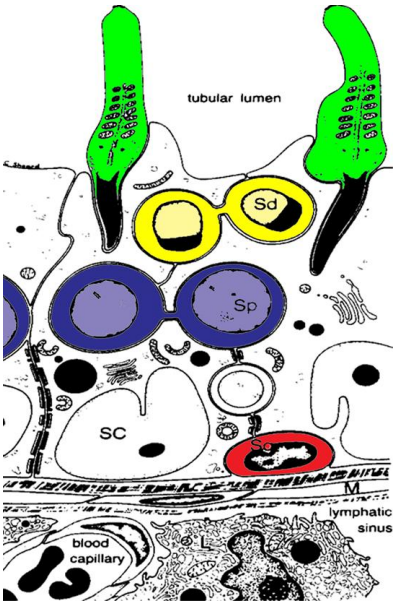
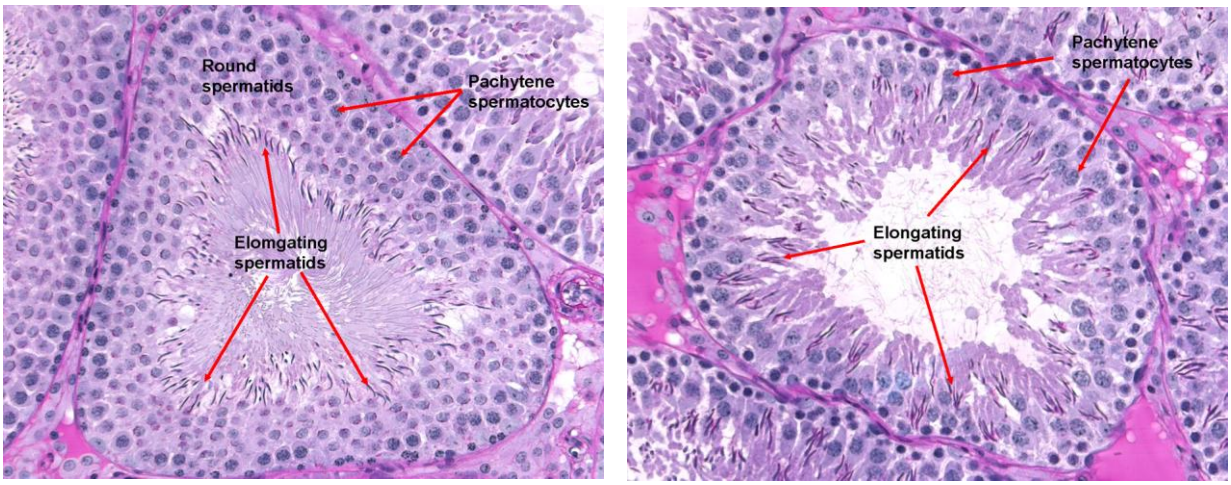


Germ cells lie in discrete layers within the seminiferous tubule supported by the cytoplasmic processes of the Sertoli cell (SC). Spermatogonia (Sg) lie on the basal lamina, spermatocytes (Sp) lie mid way in the epithelium, round spermatids (Sd) lie in an adluminal position and the elongating spermatids lie at the luminal surface with their heads embedded in Sertoli cell cytoplasmic invaginations and the tails extending into the lumen. In each tubule there are 4 generations of germ cells developing in total synchrony with one another.

The synchronous development of the 4 generations of cells results in the repetitive appearance of specific cell associations which are referred to as stages of the spermatogenic cycle. 14 such cell associations have been described in the rat and are referred to as stages I-XIV of the spermatogenic cycle.



Normal appearance and cell types in a stage VII tubule (left) and a stage XII tubule (right)
The morphological appearance of tubules in the first half of the cycle (stages I-VIII) is different from those in the second half of the cycle (stages IX-XIV). Placing the tubules into the first (early) or second (late) half of the cycle is the first step in identifying the precise stage of spermatogenesis. This can be done at low power on the microscope.

Early stage tubules have two generations of spermatids: round spermatids and mature, elongating spermatids whereas the second half of the cycle only has one generation of spermatids which are in the early phase of elongation.
In the above stage VII tubule note the layers of round spermatids plus the adlumenal layer of elongate spermatids. Also note the single layer of small pachytene spermatocytes lying beneath the round spermatids. The few small dark staining cells at the base of the tubule are preleptotene spermatocytes.
In the late stage (XII) tubule there is only one generation of spermatids and these are elongating. The other major cell types consist of multiple layers (representing one generation) of large pachytene spermatocytes (compare with the size and appearance of the pachytene spermatocytes in the early stage tubule). The dark staining cells lying beneath the pachytene spermatocytes are leptotene/zygotene spermatocytes that have developed from the preleptotene spermatocytes seen in the early stage VII tubule.

The spermatogenic cycle of the rat can be thought of as a 14 frame, time-lapse film of germ cell development. Each frame, represented by a "*stage"* is fractionally different from the frame before, as each generation of germ cells develops with time. **It is essential**

for the pathologist to have a basic understanding of the spermatogenic cycle and to be familiar with the cellular makeup of the different stages of the spermatogenic cycle in order to be able to detect subtle changes in the testes, particularly those associated with endocrine disruption, since they are characteristically cell and stage specific. It is beyond the scope of these guidelines to review the spermatogenic cycle, and how to recognize the cell associations, but the reader should refer to the following comprehensive reviews on the subject (Leblond and Clermont, 1952; Russell, 1990; Creasy, 1997; Creasy, 2002).

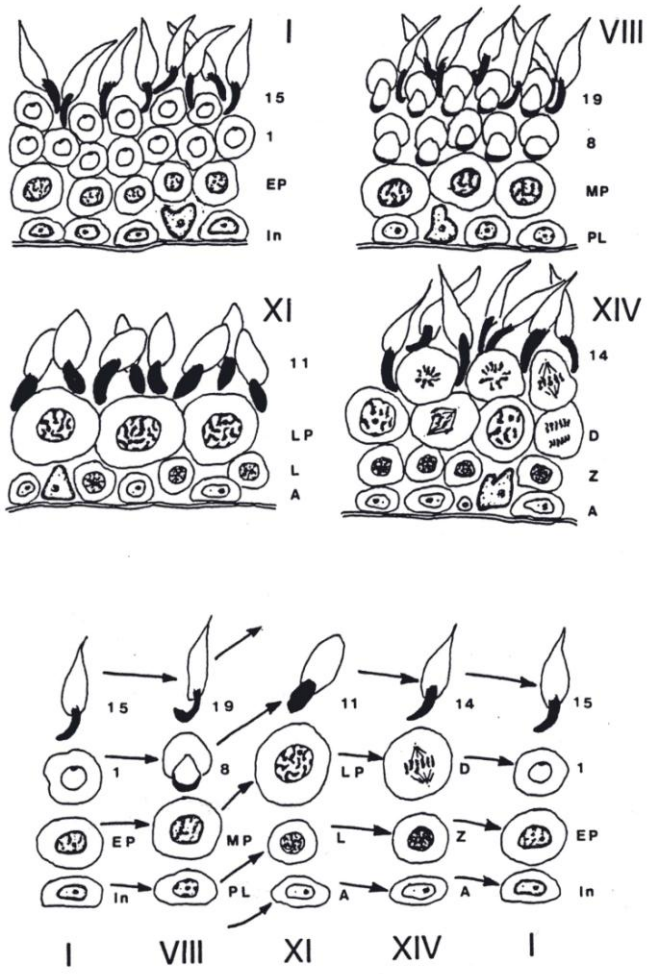


Illustration of the cell associations comprising four of the fourteen stages of the spermatogenic cycle. During the transition between stage I and VIII the round spermatids are progressively forming an acrosomic cap, as they develop from step 1 to step 8 of spermiogenesis, the early pachytene spermatocytes (EP) enlarge as they move into mid pachytene (MP), and the intermediate spermatogonia (In) complete a number of mitotic divisions to become preleptotene spermatocytes. During stage VIII, the fully mature (step 19) elongated spermatids are released into the lumen. At this point a newly committed generation of spermatogonia (A) begin dividing and displace the newly formed preleptotene spermatocytes (PL) off the basal lamina. By stage IX, the round spermatid population has begun to elongate so that by stage XI there are step 11 spermatids that have an obvious elongated profile. The pachytene spermatocytes have become very large and enter late pachytene (LP), and the preleptotene spermatocytes move into leptotene phase (L). During stage XIV the primary and secondary meiotic divisions take place and transform the large pachytene spermatocytes into new step 1 spermatids while zygotene spermatocytes enter early pachytene. It can be seen that the cellular makeup of the stage following meiotic division (stage I) is exactly the same as the cell association that the cycle began with, the difference being that one generation (of sperm) has been released and a new generation (of spermatogonia) has joined, and the rest of the cells are 14 days older and have moved up a layer.

Testosterone Biosynthesis

The major androgenic steroid testosterone is synthesized primarily in the Leydig cells and has both intratesticular effects (on spermatogenesis) and peripheral effects (on accessory sex organs as well as non-reproductive organs such as muscle, bone, skin and bone to name a few). While there is also significant testosterone synthesis in many peripheral tissues, it is beyond the scope of this review and will not be discussed further. The concentration of testosterone within the testis is very much greater than in the systemic circulation. For example, levels of the steroid in the testicular interstitial fluid can be up to 100-fold higher than in the plasma, and the concentrations in the two compartments are not directly proportional to one another. Therefore sampling plasma levels of testosterone does not provide a measure of testicular testosterone levels. Although these high intratesticular testosterone levels may be required to quantitatively maintain maximum spermatogenic potential, **qualitatively** normal spermatogenesis can be maintained with much lower intratesticular concentrations.

Testosterone is not stored within the Leydig cell, it is secreted into the interstitial fluid as it is synthesized. From here it is either i) taken up by the Sertoli cells and bound to androgen binding protein, which is then secreted by the Sertoli cell and transported through the seminiferous epithelium into the seminiferous tubule fluid and on into the epididymis or ii) diffuses into the interstitial capillaries where it binds quickly to albumin for transport through the body, where it has wide ranging effects on all other tissues of the body.

The major stimulus for testosterone production comes from blood levels of luteinizing hormone (LH) from the pituitary. Feedback inhibition of LH and hypothalamic gonadotrophic releasing hormone (GnRH) is mediated through circulating levels of testosterone and its metabolites, dihydrotestosterone (DHT) and oestradiol. Aromatization of testosterone to oestradiol takes place within the testis (indeed, oestradiol is critically important for normal testis function), and also in many peripheral tissues such as adipose tissue and the CNS, whereas conversion to DHT occurs largely in androgen dependent tissues such as the epididymis, prostate and seminal vesicles.

Maintenance of spermatogenesis

The main known effects of testosterone in supporting spermatogenesis are to stimulate seminiferous tubule fluid production by the Sertoli cell, regulate release of the mature spermatids from the Sertoli cell (spermiation) and to support the development of pachytene spermatocytes and later germ cell types through stage VII of the spermatogenic cycle. This spermatogenic support appears to be mediated by the secretion of several specific proteins from the Sertoli, peritubular and germ cells whose secretion is dependent, both on testosterone and a full complement of germ cells. Selective depletion of any of the different populations of cells (spermatocytes, round or elongating spermatids, but particularly the latter) from these stages will differentially alter (reduce or increase) the secretion of each of the androgen regulated proteins. Regulation of spermatogenesis is therefore an extremely complex cascade of cell-cell interactions with the Leydig cells supporting germ cell development through the effects of testosterone on

Sertoli and peritubular cell protein secretion but with the germ cells programming the response of these target cells to the testosterone. While the Leydig cells secrete several dozen other paracrine factors which are known to bind to receptors in the Sertoli cells, the functions of these neighbor-modulators are still being determined.

Efferent Ducts and Epididymis

Function, physiology and regulation

There are three major functions of the efferent ducts and epididymis: 1) reabsorption of seminiferous tubular fluid, 2) sperm modification and maturation and 3) sperm storage.

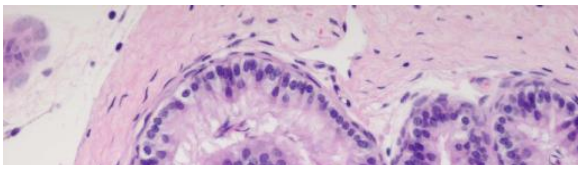
Sperm are transported from the testis in seminiferous tubular fluid that is secreted by the Sertoli cell. Over 98% of this fluid is reabsorbed as it passes through the rete, efferent ducts and initial segment of the epididymis. Oestrogen is a major regulatory factor in the resorptive process, and this function can be significantly disrupted by antioestrogens.

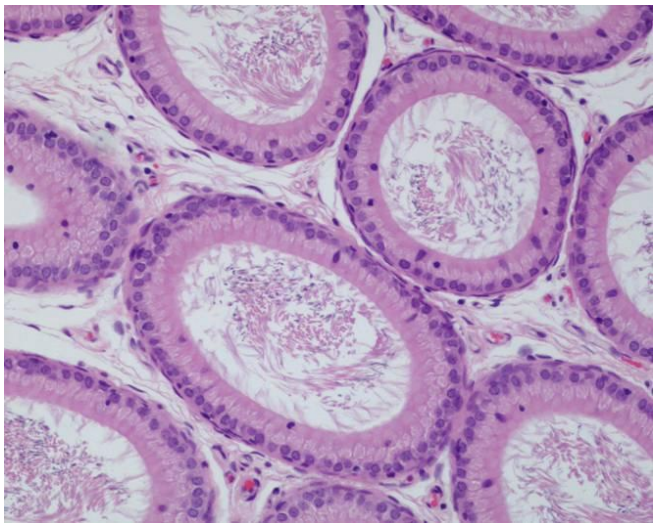
When sperm are released from the testis they are neither motile nor capable of fertilizing an oocyte. By the time they reach the cauda epididymis, they have acquired progressive forward motility and fertilizing ability. These properties are conferred by secretions of the epithelial cells in the caput and corpus epididymis, which adsorb onto the sperm, modifying their membrane function. The maturing sperm also lose their cytoplasmic droplet in the cauda epididymis. Once in the cauda, the sperm are stored, immobilized and surrounded by a glutinous glycoprotein matrix (containing the secreted protein immobilin) until ejaculation occurs.

Structure

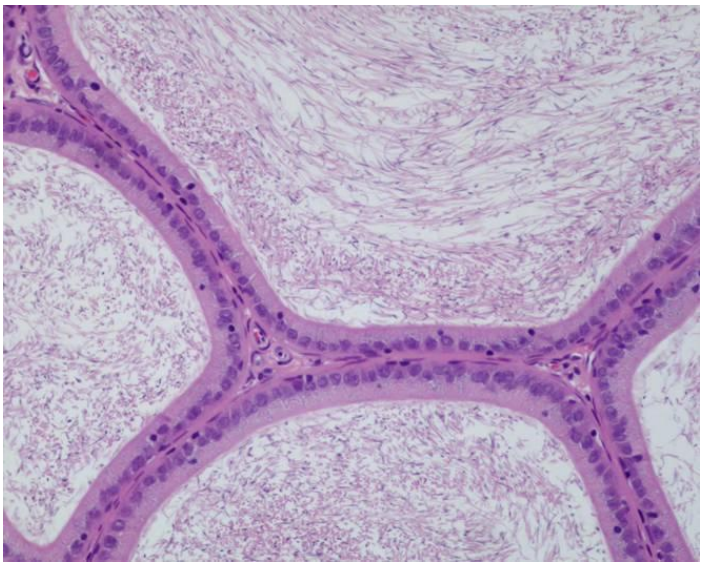
The efferent ducts comprise 7-13 ducts that link the rete testis with the initial segment of the epididymis. They are located in the epididymal fat pad and unfortunately, are generally discarded at necropsy. However, they are potentially an important target site for chemicals that disrupt oestrogen synthesis or block oestrogen receptors. For example, toxicity in these cells can reduce fluid resorption which increases the hydrostatic pressure in the testis, which will eventually shut down spermatogenesis. They are sometimes sampled when a gross observation is noted, such as discoloration or nodule or mass. If macroscopic observations are recorded in the epididymal fat pad of treated animals the pathologist should be aware of the potential for this to be evidence of endocrine disruption and recommend sampling of the epididymal fat pad from all animals.

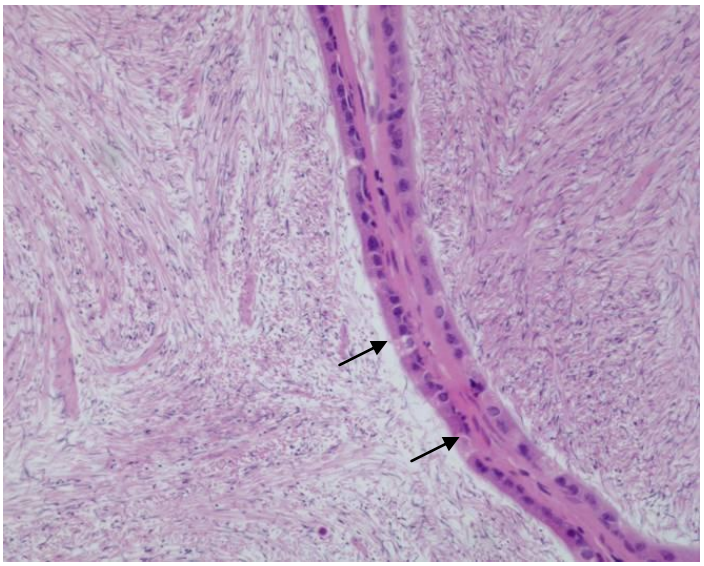
The normal histological appearance of the efferent duct is characterized by a pale staining tall cuboidal epithelium which is covered by microvilli. The multiple ducts coalesce to form a single duct which leads into the initial segment of the epididymis. The epididymis comprises a single, convoluted tube which is approximately 180 cm long in the rat and the cellular makeup, epithelial height, ductal diameter and sperm density of the epididymis all vary depending on location. Changes in endocrine status will have different impacts on different regions of the epididymis depending on the hormone (oestrogen or androgen) that is disrupted.





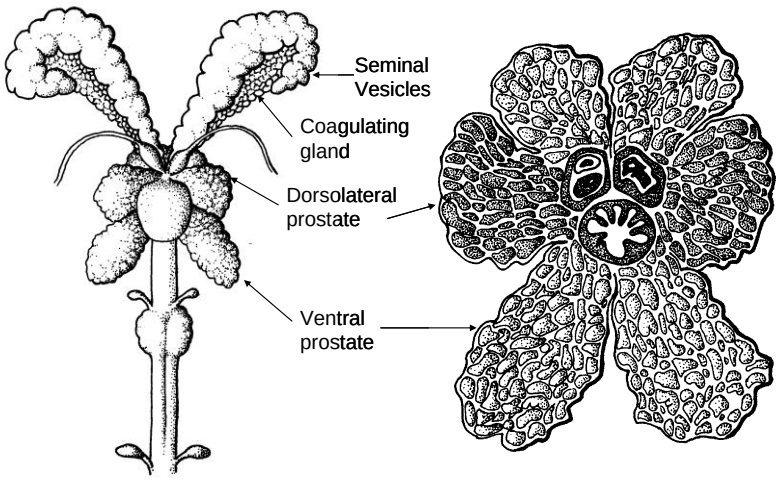
The function as well as the cellular make up of the efferent ducts and different parts of the epididymis vary. The efferent ducts, which are present in the epididymal fat pad, and the initial segment of the epididymis are made up of tall pale epithelial cells that reabsorb over 98% of the seminiferous fluid.





The caput epithelium (left) secretes protein that is important in sperm maturation while the cauda epithelium (right) reabsorbs protein and the cytoplasmic droplet that is shed from the sperm during epididymal transit. The endocytic clear cells (arrowed) are a prominent cell type of the distal corpus and cauda epithelium, which stain intensely with PAS and which become larger and more numerous when there is increased cell debris in the ductal lumens.

Accessory Sex Organs



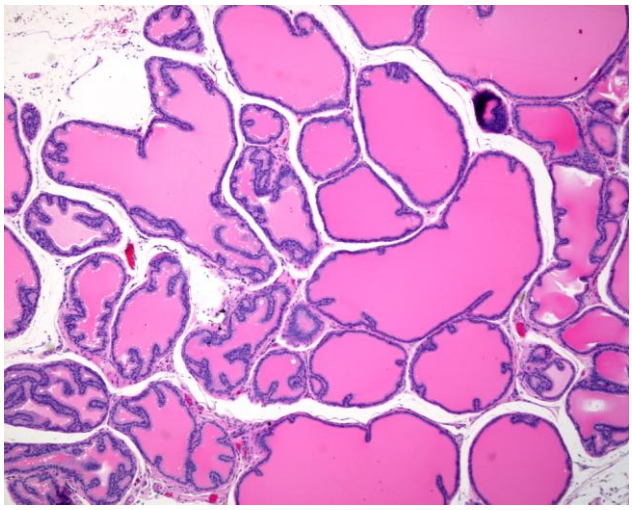
The accessory sex organs in rodents include the seminal vesicles, prostate and coagulating gland. They are located along the route of the urethra as it relays sperm from the vas deferens out through the penis. The glands secrete a variety of complex fluids that i) transport the sperm, ii) neutralize the acid environment of the female tract, iii) provide metabolic substrates for the sperm, and iv) combine to form the vaginal (copulatory) plug. Their structure is typical of active exocrine secretory glands, although the characteristics of the individual secretions are markedly different. Since the secretory activity of the accessory sex glands is extremely sensitive to androgen levels, weight change and altered secretory activity in the prostate and seminal vesicle can be used as a good, and relatively rapid,integrated indicator of altered circulating androgen levels

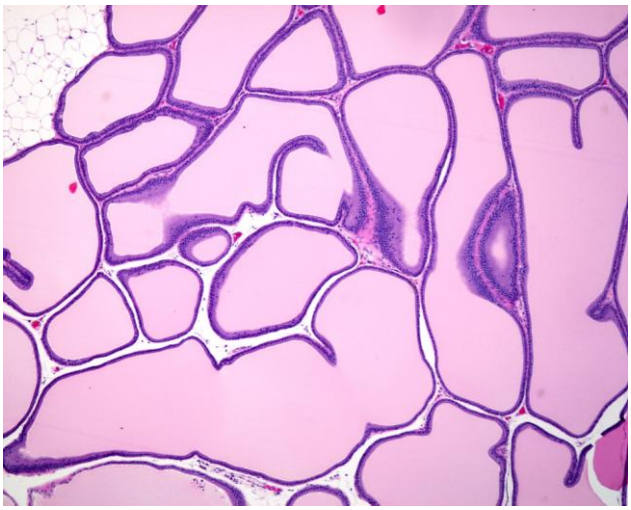
Prostate and Coagulating Gland

The prostate forms multiple lobes around the urethra. It is a compound tubuloalveolar gland that secretes a colorless serous fluid into the urethra through a number of ducts. In the rat, a discrete pair of ventral lobes and a smaller group of dorsal and lateral lobes (dorsolateral lobes) are situated at the neck of the bladder. A pair of anterior lobes, otherwise known as the coagulating glands, is situated closely adjacent to and running up the medial aspect of the seminal vesicle. The glandular acini are lined by a simple columnar epithelium. The prostatic fluid secretion constitutes 15–30% of the ejaculate. It is a colorless fluid rich in proteolytic enzymes (e.g., acid phosphatase). The fluid also contains relatively high levels of zinc, inositol, transferrin, and citric acid.

The comparative histopathological structure of the various parts of the prostate varies slightly with respect to staining properties of the secretions and the degree of papillary infolding of the acinar epithelium.

Increased levels of oestrogen result in acute inflammation of the acini of the dorsal prostate and this provides an important endpoint for detection of oestrogenic compounds. The ventral lobes constitute the major part of the prostate and are the lobes that are most sensitive to circulating androgen levels.

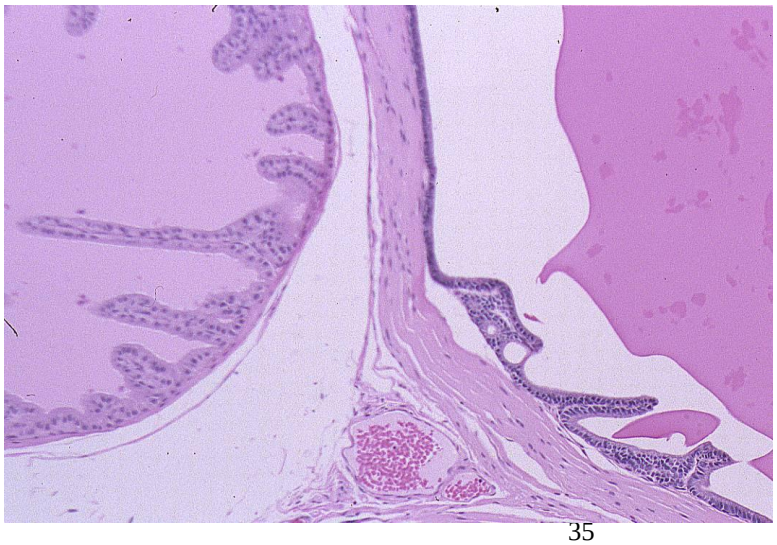




Normal dorsolateral prostate (left) and ventral prostate (right)
Note smaller acini, increased eosinophilic secretion and increased papillary infolding of the epithelium in the dorsolateral prostate. Dorsolateral prostate responds to oestrogen with acute inflammation, ventral prostate responds to low androgen with atrophy.

Seminal Vesicle

The seminal vesicles are paired elongated hollow organs filled with a yellowish-white viscous fluid. They are situated distal to the ampulla of the vas deferens and empty via the ejaculatory duct into the urethra. The mucosa has a honeycombed structure formed by complex folding to produce irregular anastomosing channels that communicate with the central cavity; thin primary folds of the mucosa also extend out into the vesicle lumen. The epithelium is composed of pseudostratified columnar cells in the mouse and simple columnar epithelium in the rat. The seminal vesicle fluid is a viscous secretion constituting 50–80% of the ejaculate. The fluid is alkaline, which is thought to neutralize the acid pH of the vagina; it contains citric acid as the major component, as well as fructose and lactoferrin. Lactoferrin is one of the sperm-coating antigens and, as its name suggests, is also involved in iron binding.



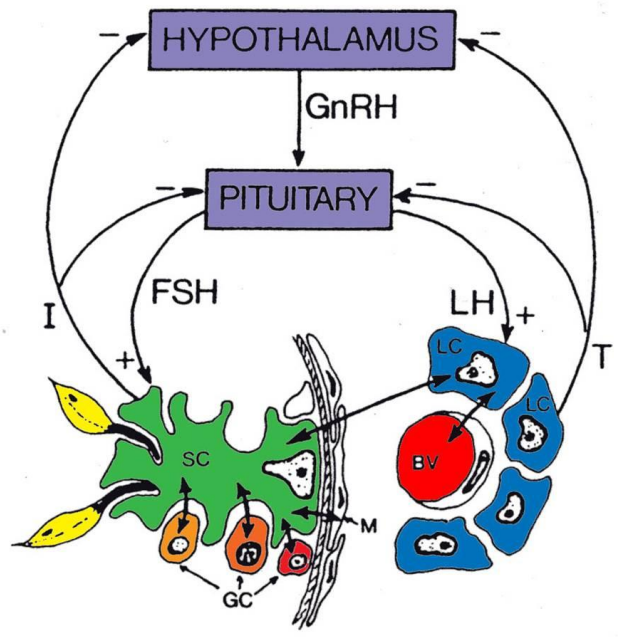
Coagulating gland (left), sometimes called the anterior prostate. Responsible for copulatory plug formation. Seminal vesicle (right). Both are androgen dependent, particularly the seminal vesicle

Hormonal Regulation of Reproductive Tissues

Regulation of spermatogenesis relies not only on the classical endocrine control involving the hypothalamic - pituitary - testicular axis, but also on the complex autocrine and paracrine interactions involving the Sertoli cells, germ cells, Leydig cells, peritubular cells, testicular macrophages and the endothelial cells of the interstitial vasculature. This is a rapidly advancing area of research which has important and pivotal implications for mechanistic investigations of male reproductive toxicity

There are different levels of hormonal regulation of the reproductive tissues. Most people are familiar with classic endocrine regulation, involving the hypothalamic-pituitary-gonadal axis but it is important to be aware that there is another tier of regulation involving paracrine interactions between neighboring cells and autocrine regulation of a cell by itself. This is particularly prevalent in the testis where regulatory peptides and growth factors, secreted by the Leydig cells, Sertoli cells, germ cells and peritubular cells, are believed to mediate local control of cellular function between the various cells or within the cell that is secreting the factor.

Endocrine/reproductive tests are largely concerned with detecting disturbances in endocrine signaling and the basic pathways involved in regulation are shown in the following diagram.



Basic endocrine pathways of the hypothalamic pituitary testis axis. GnRH is released from the hypothalamus and travels to the pituitary via the hypothalamophyseal tract where it stimulates FSH and LH release into the peripheral circulation. FSH acts on the Sertoli cells and modulates spermatogenesis while LH acts on the Leydig cell to stimulate testosterone (T) biosynthesis. Negative feedback by testosterone and inhibin (secreted by the Sertoli cell in response to FSH) down regulates LH and GnRH release from the pituitary and hypothalamus. In addition to endocrine regulation, the various cells of the testis regulate one another through paracrine pathways. This involves secretion of a multitude of peptides and growth factors which provide local control of cellular fuction between Sertoli cells (SC), germ cells (GC), peritubular myoid cells (M), Leydig cells (LC) and endothelial cells of the blood vessels (BV).

Normal Background Variation of Structure

Testes

Rat spermatogenesis is extremely regular and highly efficient such that in the normal adult rat (>10 weeks old) there are very few degenerating or depleted germ cells. However, animals that are younger than 10 weeks may show increased numbers of degenerating germ cells and partial depletion of some germ cells, particularly the elongating spermatids. although subtle changes here can be difficult to appreciate. The degree of germ cell degeneration and depletion is greater in younger rats. This factor must be taken into account when evaluating animals euthanized prior to scheduled sacrifice.

The most common background microscopic changes seen are:

- occasional atrophic tubular profiles (1-3 contracted tubules containing only Sertoli cells)
- occasional tubular vacuoles
- occasional degenerating (eosinophilic cytoplasm or syncitial) germ cells
- occasional stage XI/XII tubules with a very low number (≤3) retained step 19 spermatid heads in the basal Sertoli cell cytoplasm (spermatid retention/delayed spermiation)
- occasional stage IX-XI tubules with retained step 19 spermatids at the luminal surface (spermatid retention/delayed spermiation)
- diffuse germ cell degeneration/depletion or total tubular atrophy affecting one or both testes

The changes listed above can all be seen as incidental background findings in adult rats but they are generally infrequent. There are minor differences in the background pathology between different strains of rat and it is important to have good historical background data for the strain used in the study.

Relationship to treatment of a finding should be based on the consistency of the finding in the treated rats, the degree above background in the controls, and its dose relationship.

Epididymis

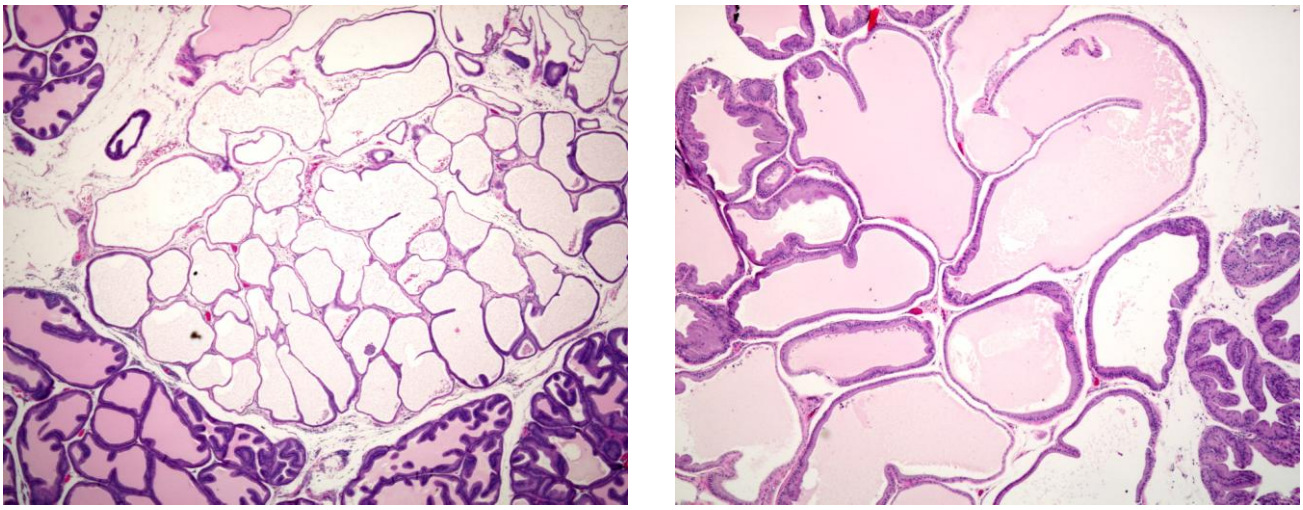
As with the testes, the epididymides of normal adult rats have very consistent morphology and have very few abnormalities. In particular, the presence of sloughed testicular germ cells and cell debris in the epididymal lumen is a very sensitive indicator of disrupted spermatogenesis in the testis and subtle disturbances of spermatogenesis are often more readily identified by changes in epididymal content than by the testicular changes. However, sloughed and degenerate testicular germ cells and cell debris are commonly seen in peripubertal rats (<10 weeks old), particularly in the cauda (the contents of which, reflects the output of the testis 1-2 weeks earlier). The diameter of the cauda epididymis and the concentration of sperm in the cauda will also be more variable in younger animals.

Be aware that the different regions of the epididymis (initial segment, caput, corpus and cauda) are all different histologically, functionally and dynamically. For example, the contents of the caput reflect sperm just released from the testis whereas the contents of the cauda reflect sperm that left the testis 4-14 days previously. This is particularly important for interpretation of potential treatment related effects.

Prostate and Seminal Vesicles

The apparent size of the seminal vesicle and the amount of secretion within the vesicle lumen can vary due to sampling procedures, so it is important to be sure that any apparent changes in size are real rather than due to leakage of fluid at necropsy or plane of sectioning. The height of the individual epithelial cells and the presence of an apical secretory vacuole are good indicators of secretory activity.

In the prostate, focal groups of acini can appear atrophic, with flattened squamous epithelium and reduced or absent secretion (Figure 9). This focal atrophy should not be mistaken for a treatment related effect, which is much more likely to appear as a diffuse change and be associated with stromal expansion.



Focal variability in epithelial height and secretory content of the ventral prostate in a control animal. This should not be mistaken for a test article related effect

Decreased body weight gains of ≥ 10% of controls results in decreased size and weight of the seminal vesicles and prostate. However, there are no changes in the weight or morphology of the testes or epididymides with decreases in body weight gain of ≤ 30% of controls (Chapin *et al.*, 1993).

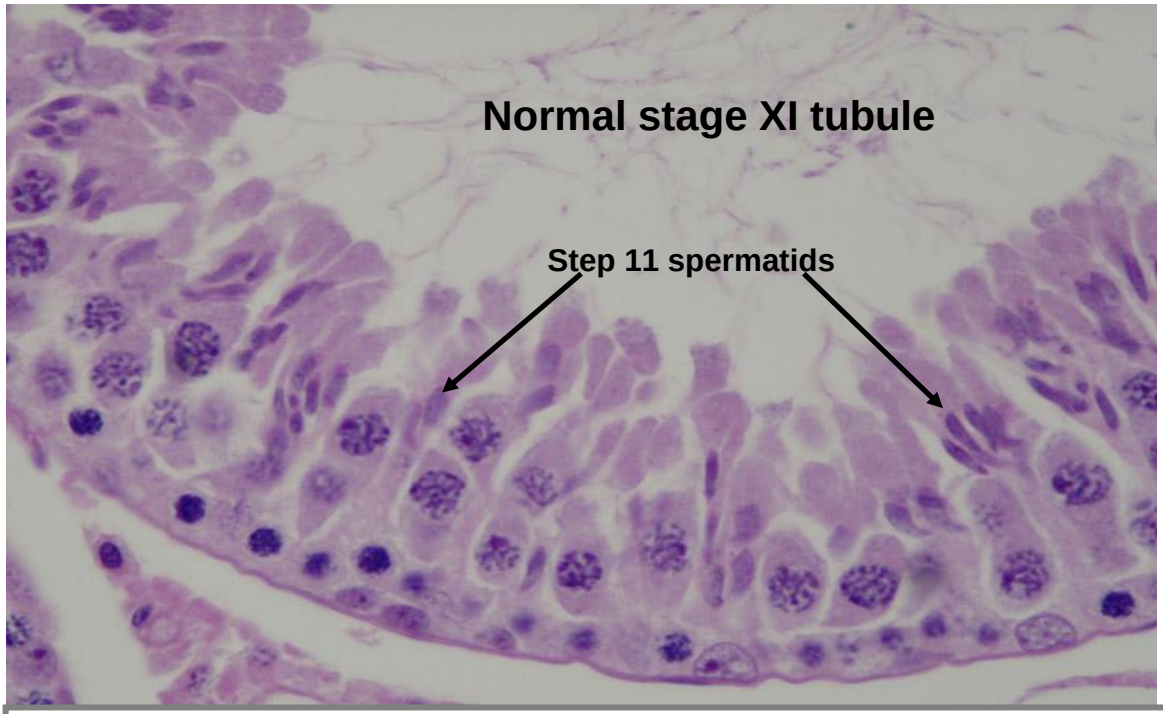
Morphologic Patterns of Hormone Disruption

Endocrine disrupting agents affecting the male reproductive system can be broadly classified into oestrogenic (those acting like a natural oestrogen), androgenic (those acting like a natural androgen), antioestrogenic (ability to suppress the action of a natural oestrogen), antiandrogenic (ability to suppress the action of a natural androgen) and steroidogenesis inhibitors (ability to suppress steroidogenesis). However, the patterns or profiles of morphological change that occur in the various tissues of the reproductive tract in response to chemicals altering oestrogen and androgen pathways do not fit so neatly into these broad categories. Interpretation of results needs to integrate the responses and structural changes in the individual tissues (testes, epididymides and accessory sex organs) to provide an overall profile of what is going on. The profile provided here describes the changes that might be expected in a 28 day dosing study such as the TG407. Longer duration dosing might produce different profiles due to the secondary effects of long-term disruption of the hypothalamic-pituitary-gonad axis (e.g. persistent increase in LH leading to Leydig cell proliferation).

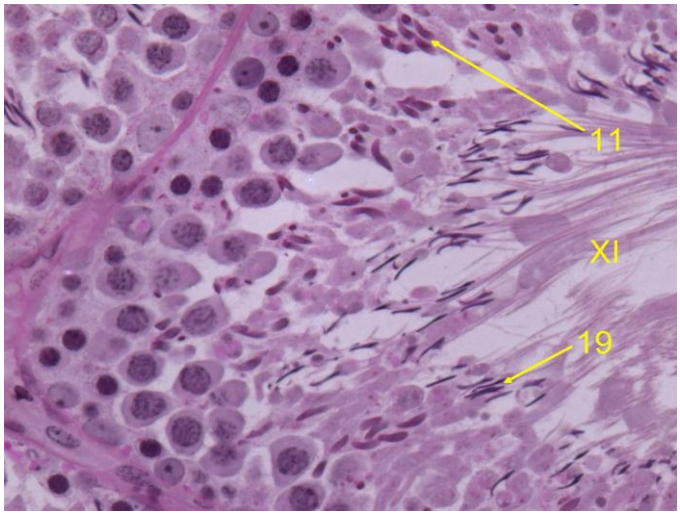
Table 1 summarizes the profile of morphologic changes expected in the testes (spermatogenesis and Leydig cell), epididymides, and accessory sex organs (prostate and seminal vesicles) in response to the major types of endocrine disruption that might occur following xenobiotic administration. The subsequent sections explain the endocrine signals that underlie the morphological profile for each of the major classes of endocrine disruption.

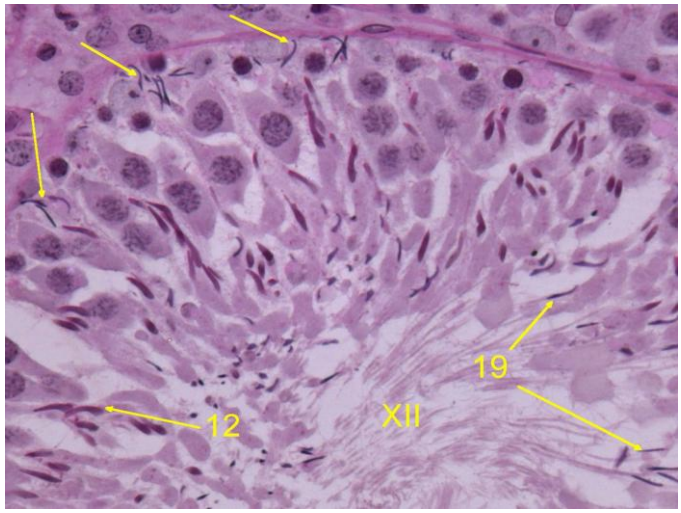
The range of responses of the various tissues to hormonal change is fairly restricted. In the prostate and seminal vesicles, the major response is increased or decreased secretory activity, which can often manifest as a weight change. In the testes, increased or decreased testosterone secretion will dictate the morphologic pattern of changes. Since stages VII and VIII of the spermatogenic cycle are the most visibly androgen dependent, these may be the only tubules to exhibit morphologic changes in response to endocrine disruption. Those changes are also restricted to degeneration of a small number of specific cells (pachytene spermatocytes and round spermatids) in those two stages of tubules and so the pathologist needs to carefully examine the testes for these specific changes. However, this cell- and stage-specific response pattern has been repeatedly demonstrated to be the most sensitive indicator of decreased testosterone levels in the testis (Russell and Clermont, 1977), so it is reasonable to begin one's search for endocrine disruption at these stages. Sperm release (spermiation) is an androgen dependant function of the Sertoli cell and this will also be disturbed by decreased testosterone levels. The disturbance results in spermatid retention (delayed spermiation). This is also a stage-specific change and is recognized by the inappropriate presence of step 19 spermatids still adhering to the adluminal surface of stage IX-XI tubules or being phagocytosed into the basal Sertoli cell cytoplasm of stage XI-XII tubules. **These histopathologic changes are far more sensitive than changes in testis weight.** With prolonged or marked reductions in testosterone levels, a more generalized degeneration and depletion of elongating spermatids will develop along with a contraction of tubular lumen size, due to reduced production of seminiferous tubule fluid by the Sertoli cells.

Secretion of seminiferous tubule fluid by the Sertoli cell is an androgen dependant function that is regulated by the presence of elongating spermatids.



Normal stage XI tubule with only one generation of elongating (step 11 spermatids)





Stage XI tubule (left) with 2 generations of elongating spermatids: step 11 spermatids (which should be present) plus the aberrant step 19 spermatids which failed to be released at stage VIII.
Stage XII tubule (right) with step 12 spermatids (which should be present). The lesion here is a few remaining step 19 spermatids at the lumen plus many phagocytized step 19 spermatid heads in the basal Sertoli cell cytoplasm.

Decreased Steroidogenesis and Antiandrogens

Decreased androgen signal can be caused by various mechanisms. Chemicals can cause decreased production of testosterone through direct inhibition of steroidogenesis or indirectly through the hypothalamic pituitary axis. It is also important to be aware of the fact that decreased body weight gain or reduced food intake as well as stress will result in decreased testosterone due to decreased GnRH release. Decreased metabolism of testosterone to the more potent and long lived androgen dihydrotestosterone through inhibition of 5 alpha reductase, or decreased metabolism to oestradiol through aromatase inhibition will result in changes indicative of decreased androgen stimulation. Androgen receptor antagonists that inhibit ligand binding to the androgen receptor (antiandrogens) will also decrease the androgen signal. However, the morphologic response of the various reproductive tissues is slightly different with each mode of action.

Decreased testosterone biosynthesis

This is the most commonly encountered response of the reproductive tissues to hormone disruption. It can occur through direct inhibition of steroidogenesis in the Leydig cell (e.g. ketoconazole) or through reduced stimulation of the Leydig cell by LH (GnRH antagonism) or through decreased numbers or sensitivity of LH receptors on the Leydig cell (e.g. through hypoprolactinemia).

The overall result will be decreased secretion of testosterone which will result in decreased spermatogenesis, varying degrees of Leydig cell atrophy (which may or may not be qualitatively detectable), and decreased size and secretory activity of the epididymis and accessory sex organs which are more readily detected by organ weight change than by histopathology (O'Connor *et al.*, 2002b).

Decreased metabolism of testosterone to dihydrotestosterone (DHT)

The primary effector androgen in the epididymis, prostate and seminal vesicles is DHT, which is produced by the action of intracellular 5-alpha reductase on testosterone that arrives at these tissues in the peripheral circulation and in the seminiferous tubule fluid exiting the testis. DHT is synthesized within the target tissues. Testosterone is the primary androgen for testicular spermatogenesis, so inhibition of DHT formation will have no morphological effects on spermatogenesis, but will cause decreased weight of the epididymis, prostate and seminal vesicles. Depending on the severity of the response, this may or may not be detectable microscopically.

Androgen receptor antagonists

Androgen action on effector tissues/cells relies on the interaction of the ligand with the androgen receptor. If this is blocked then the tissue/cell cannot respond to normal levels of circulating androgen. The main effect of androgen receptor antagonists is seen as weight loss in the epididymis and accessory sex organs which, depending on severity, may or may not be detectable microscopically (O'Connor *et al.*, 2002a). Mild effects on spermatogenesis including spermatid retention and degeneration of round spermatids and pachytene spermatocytes may be seen in the testes.

In addition to the effects on the reproductive tissues, blockade of the androgen receptor in the hypothalamus and pituitary will result in inhibition of the normal negative feedback of testosterone. This will result in increased LH secretion which will increase testosterone secretion from the Leydig cell which may be reflected by morphologically detectable Leydig cell hypertrophy/hyperplasia. Thus, the picture produced by a receptor antagonist is that of target tissue atrophy in the presence of elevated blood levels of the hormone.

Androgenicity

The response of the reproductive tissues to androgens can be confusing. In the testis, the response is the same as that seen with decreased testosterone secretion, whereas the prostate and seminal vesicles respond with a hypertrophic response. The differential response is due to the widely different concentrations of testosterone within the testis versus those in the peripheral circulation. Extremely high intratesticular levels of testosterone are required for maintenance of spermatogenesis, whereas very low circulating levels are required for maintenance of secretory function in the accessory sex glands.

Administration of androgens to males results in inhibition of GnRH and LH at the level of the hypothalamus and pituitary, through normal negative feedback mechanisms. This results in decreased testosterone secretion from the Leydig cells, which produces changes in spermatogenesis that are characteristic of low testosterone (see above). However, the secondary sex organs respond to the exogenously administered androgen with enlargement and increased secretion (O'Connor *et al.*, 2000).

In the testis, the dose response for spermatogenic disruption to exogenous androgen is generally inverse from what is usually expected (lower doses yield lower testis weights), while the enlargement of the secondary sex organs is positively correlated with dose. This is explained by the fact that small amounts of exogenous androgen will effectively inhibit pituitary LH release and thus reduce testosterone secretion but will be inadequate to replace the normally high levels of intratesticular testosterone. As the dose of exogenous testosterone increases, it still inhibits endogenous testosterone secretion but the higher administered levels are capable of partially replacing the low levels of intratesticular testosterone and thus partially restoring spermatogenesis.

Oestrogenicity

Oestrogenic chemicals act in the male reproductive tract, at least in part by inhibiting testosterone secretion from the Leydig cell. This is partly due to a direct effect on the Leydig cells, but is mainly mediated through oestrogen acting as a negative feedback molecule on GnRH and LH release at the level of the hypothalamus and pituitary. The profile of changes will be the same as for chemicals inhibiting testosterone secretion, (Cook *et al.*, 1998; Yamasaki *et al.*, 2002). An additional finding for oestrogenic chemicals, is acute inflammation of the dorsolateral prostate, an effect that may be mediated through oestrogen induced increase in prolactin levels (Tangbanluekal and Robinette, 1993)

Antioestrogens and Aromatase Inhibitors

Oestrogen is an important positive regulator of fluid reabsorption in the rete testis, efferent ducts and initial segment of the epididymis. Over 95% of the fluid produced by the seminiferous tubules is reabsorbed in this region and the major regulatory molecule opf this process appears to be oestrogen, interacting with ER alpha receptors that are highly expressed in this region. Antioestrogens that block the oestrogen receptor have been shown to inhibit this fluid reabsorption, producing elevated backpressure and dilated efferent ducts (which are not routinely sampled or examined) as well as dilated lumens in the seminiferous tubules, due to back pressure caused by the increased fluid in the efferent ducts. The dilated tubules and increased fluid in the testes are generally reflected by an **increase** in weight of the testes. Initially, tubular dilation occurs in the presence of normal appearing spermatogenesis, but if the dilation is prolonged and/or severe, the excess pressure in the tubules can lead to total loss of all germ cells from all tubules, leaving a dilated seminiferous tubule lined only by Sertoli cells (Oliveira *et al.*, 2002).

Oestrogen is produced in germ cells and Leydig cells from testosterone by the action of aromatase, which is present in these two cell types. It is also produced in various peripheral tissues, for example adipose tissue, which contains aromatase and converts circulating testosterone locally. Aromatase inhibitors have been shown to reduce circulating levels of testosterone and DHT, and cause spermatid retention in the testis and reduced weight of the accessory glands (O'Conner et al 2002).

Table 1: Morphological patterns of changes associated with various endocrine disruption pathways

Endocrine disruption mechanism	Spermatogenesis	Leydig cell	Epididymis	Accessory sex glands
Marked decrease in testosterone biosynthesis LHRH receptor antagonism Strong oestrogen receptor agonism	**1.** Stage IX-XII spermatid retention **2.** Stage VII/VIII degenerate round spermatids and spermatocytes **3.** progressive degeneration and depletion of elongating spermatids 4. Slight reductions in the numbers of round spermatids and pachytene spermatocytes (all stages)	Atrophy	1. Organ weight decrease 2. Decreased content of sperm in the epididymis and sloughed testicular germ cells 3. Ductal atrophy of the epididymis 4. Epithelial apoptosis in specific segments of the caput epididymis (sometimes, but not always seen)	1. Organ weight decrease 2. May be detectable as decrease in acinar/vesicle size and amount of secretion
Slight decrease in testosterone biosynthesis Weak oestrogens	1. No change or: 2. Stage IX-XII spermatid retention 3. Stage VII/VIII degenerate round spermatids and spermatocytes	No change	1. Organ weight decrease 2. Sloughed testicular germ cells	1. Organ weight decrease 2. Not detectable morphologically
Androgen receptor antagonism Aromatase inhibition	1. No change or: 2. Stage IX-XII spermatid retention 3. Stage VII/VIII degenerate round spermatids and spermatocytes	No change or: Hypertrophy/ hyperplasia	1. Organ weight decrease 2. Sloughed testicular germ cells	1. Organ weight decrease 2. May be detectable as decrease in acinar/vesicle size and amount of secretion
Androgen receptor agonism (shows reverse dose relationship for spermatogenesis)	**1.** Stage IX-XII spermatid retention **2.** Stage VII/VIII degenerate round spermatids and spermatocytes **3.** progressive degeneration and depletion of elongating spermatids 4. Slight reductions in the numbers of round spermatids and pachytene spermatocytes (all stages) accompanied by the presence of sloughed germ cells in the epididymal lumen	No change or: Atrophy	1. Organ weight decrease 2. Decreased content of sperm in the epididymis and sloughed testicular germ cells 3. Ductal atrophy of the epididymis	1. Organ weight increase 2. May be detectable as increase in acinar/vesicle size and amount of secretion
Strong oestrogen receptor antagonists	1. Increased testis weight 2. Increased tubule lumen diameter 3. Variable progression to tubular atrophy	No change	1. Dilation of efferent ducts (if sampled) 2. No change in epididymis (with dilated testis tubules) 3. Aspermia and ductal atrophy (with atrophic testis tubules)	No change

Recommended Terminology and Severity Grading for Histopathological Findings

Introduction

The changes in spermatogenesis associated with endocrine disruption are often subtle, cell-specific and stage-specific. The terminology used to describe such changes needs to be sufficiently detailed to provide the reader with enough information to be able to recognize that the changes are characteristic of, or consistent with endocrine disruption.

Other changes such as reduced sperm and sloughed testicular cells in the epididymis or reduced size and secretion in the accessory sex organs are relatively non-specific and a more general nomenclature is adequate.

Table 2 provides recommended terminology and grading for recording the most common changes seen in the male reproductive tract in response to endocrine disruption. This is not provided as a comprehensive list of all findings that will be seen in the testis, only a focused list on those most commonly encountered with endocrine disruption.

Histopathological Term	**Diagnostic Criteria**
Testes	
Spermatid retention: stages IX-XII	Presence of step 19 spermatids at the luminal surface or phagocytized in the cytoplasm of stage IX-XII tubules
Degenerate round spermatids and spermatocytes (stage VII/VIII)	Presence of occasional degenerating round spermatids and/or pachytene spermatocytes in stage VII/VIII tubules
Degeneration and depletion of elongating spermatid	Partial or generalized degeneration and depletion of elongating spermatids (step 11- step 19 spermatids, present in stages IX-VIII)
Depletion round spermatids and pachytene spermatocytes	Partial depletion (often accompanied by sloughing into the lumen) of round spermatids and pachytene spermatocytes (affects all stages). This is generally only seen concurrent with significant loss of elongating spermatids.
Tubular degeneration/atrophy	Non-specific degeneration and depletion of germ cells from tubules with no cell or stage specificity. May be partial or total loss of germ cells from a tubule. May affect a small or large proportion of tubules.
Leydig cell atrophy	Decreased size/number of Leydig cells.
Leydig cell hypertrophy/hyperplasia	Increased size/number of Leydig cells.
Tubular dilation	Increased luminal diameter of seminiferous tubules (generally with normal appearing spermatogenesis
Rete dilation	Increased luminal diameter of rete testis
Epididymides	
Lumenal sloughed germ cells/cell debris	Presence of immature testicular germ cells or cell debris in the luminal contents of the epididymis
Reduced sperm content	Reduced volume/numbers of sperm in the lumen of the head or tail (or both)
Prostate	
Epithelial apoptosis	Increased number of apoptotic cells, may be seen as an early event in atrophy
Acinar atrophy (specify which lobe if there is a differential effect)	Decreased size and secretory content of the prostatic acini.
Acute/subacute inflammation (specify which lobe if there is a differential effect)	Presence of an acute/subacute inflammatory infiltrate (acinar and/or interstitial) which affects dorsolateral prostate with oestrogen agonists
Seminal Vesicles	
Eepithelial apoptosis	Increased number of apoptotic cells, may be seen as an early event in atrophy
Atrophy	Epithelial atrophy, contraction of vesicle size and decreased secretory content

Severity Grading

A general guide for severity grading of spermatogenic changes in the testes relies on the numbers of tubules affected (Table 2). This grading scheme works well for changes which are not cell or stage specific, but for changes which only affect a single cell type e.g step 19 spermatids, and only affect a few stages e.g stages IX-XI, the system is not practical. Guidance for these situations is provided in Table 3.

For findings in the epididymis and accessory sex organs, conventional grading criteria ranging between Grade 1= just detectable above control levels, to Grade 5 = almost all cells or all of the tissue affected by the change are appropriate.

Table 2: Recommended grading system for non-specific changes in the testis

Grade/severity	Number of tubules affected
Grade 1 (minimal)	<10 %
Grade 2 (slight)	11-25 %
Grade 3 (moderate)	26-50 %
Grade 4 (marked)	51-75 %
Grade 5 (severe)	76-100 %

Table 3: Recommended grading system for endocrine related cell- and stage- specific findings, likely to be encountered in the testes of a 28 day TG407 study

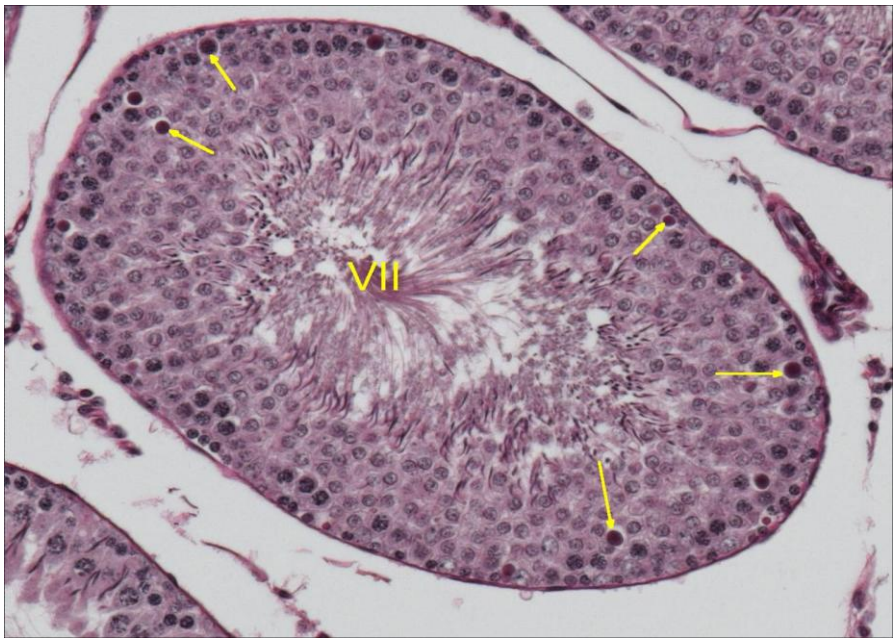
Histopathological Term	Severity Grading
Testes	
Spermatid retention: stages IX-XII	This is a subtle change that generally only affects a proportion of spermatids and a proportion of the stage IX-XII tubules. Grade 1 = just detectable above control levels Grade 2 = consistently present in a high proportion (>50%) of stage IX-XII tubules Grade 3 = prominent retention and easily detectable even at low magnification
Degenerate round spermatids and spermatocytes (stage VII/VIII)	This change only ever affects a very small number of cells (maybe 2 - 6 cells per tubular profile) Grade 1 = just detectable above control levels Grade 2 = consistently present in a high proportion (>50%) of stage VII/VIII tubules
Degeneration and depletion of elongating spermatid	Depending on the severity and duration of testosterone depletion, this change can vary from just detectable in a few stages to most cells affected in all stages. Grading should reflect this range i.e. Grade 1 = just detectable in a small proportion of tubules Grade 5 = absence of most elongating spermatids in most tubules
Depletion round spermatids and pachytene spermatocytes	This change generally only affects a small proportion of the total number of round spermatids and pachytene spermatocytes Grade 1 = just detectable above control levels Grade 2 = slightly reduced numbers of cells in many tubules
Tubular degeneration/atrophy	Use the non-specific terminology in Table 2
Leydig cell atrophy	Often difficult to appreciate any change due to the variability of Leydig cells in normal testes: Grade 1 = consistently smaller than controls but some cytoplasm still present Grade 2 = negligible cytoplasm present with small inactive appearing nuclei
Leydig cell hypertrophy/hyperplasia	Often difficult to appreciate any change due to the variability of Leydig cells in normal testes: Grade 1 = just detectable above control levels Grade 2 = consistent enlargement of cells and increased total volume of Leydig cells Grade 3: prominent enlargement/hyperplasia and easily detectable at low magnification
Tubular dilation Rete dilation	This can be a subtle or prominent change that generally affects tubules diffusely Grade 1 = just detectable above control levels Grade 2 = consistently present in a high proportion of tubules Grade 3 = prominent and easily detectable

Critical aspects of histopathological evaluation

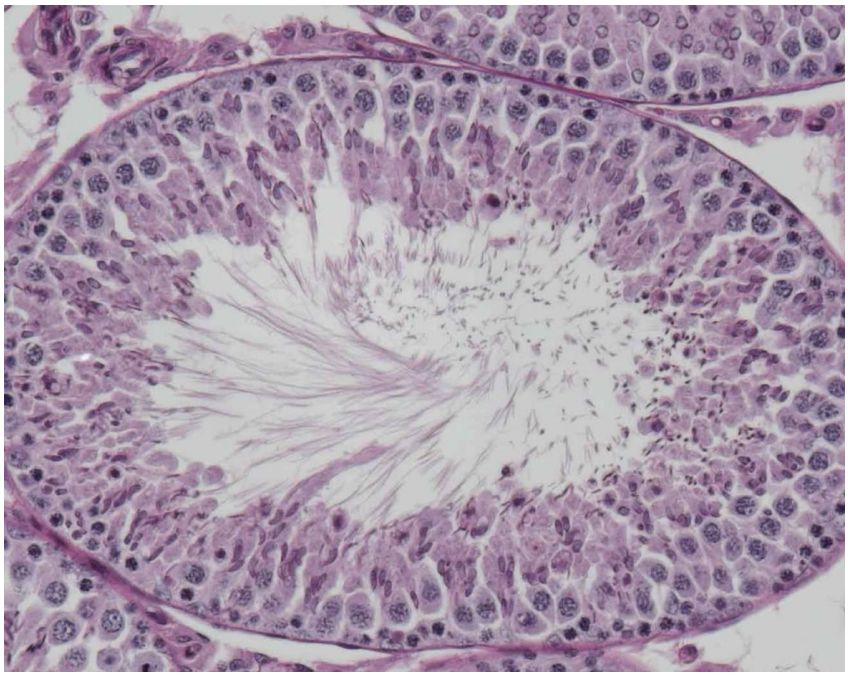
Identifying the histopathologic consequences of severe endocrine disruption in the male reproductive system is not difficult. Marked changes in organ weights of the various tissues are accompanied by significant morphologic changes in the same tissues. Detection of weak endocrine disrupting activity is much more difficult because the weight changes, and more importantly the histopathologic changes, are much more subtle and require the pathologist to have a detailed knowledge of spermatogenesis as well as the cell and stage specific changes that are characteristically associated with hormonal perturbation in the reproductive system. The more subtle the changes, the more important it is to have ideal fixation, consistent trimming of tissues and an excellent knowledge of the background variation of the normal structure and function of the tissues being examined. The TG407 protocol utilizes only 5 animals/sex/group and so weak endocrine disruptors may produce subtle changes in only a proportion of those few animals; this makes overinterpretation of findings a real risk. The following points are provided to aid the investigator in deciding what is "within normal range" and what the most sensitive indicators of endocrine disruption are.

Microscopic Finding	**Sensitivity of the finding as an endpoint of endocrine disruption**	**Variability in normal animals**
Testes		
Spermatid retention	One of the earliest and most sensitive indicators of low intratesticular testosterone, but may also be seen with other mechanisms of testicular toxicity. Needs to be interpreted in conjunction with other endpoints.	Occasionally seen in control testes at the lumen of stage IX-XI tubules. Stage XII tubules may have 1-3 retained spermatids in basal cytoplasm.
Stage VII/VIII degeneration of round spermatids and pachytene spermatocytes	Very specific and sensitive marker of low intratesticular testosterone. Only a few cells may be affected in any given tubular profile, but the cell- and stage-specificity is critical and highly diagnostic	Not generally seen as a background finding in control animals.
Degeneration/depletion of elongating spermatids	This is the end stage lesion of low intratesticular testosterone. It can also be seen with other testicular toxicants, so when present, the organ weights of the seminal vesicles and prostate should be examined for reduction, confirming low circulating androgen.	This may be seen at a low level in peripubertal rats that have not reached full spermatogenic potential. If animals are terminated prior to scheduled termination, this may reflect age rather than toxicity
Epididymis		
Sloughed germ cells	This is a very sensitive indicator of spermatogenic disturbance in the rat and even very minor disturbances in spermiation or spermatogenesis can be reflected by minimal increases in the numbers of sloughed cells and cell debris. If any increase is seen, go back and carefully re-examine the testis for changes. The position of the cells in the epididymis (caput versus cauda) will also provide information on how long the spermatogenic disturbance has been occurring	The background level of sloughed cells in the epididymis is very low in control adult rats but will be increased in peripubertal animals (e.g. animals terminated prior to scheduled termination). The same will be true of the amount of sperm in the cauda epididymis, which will be decreased in slightly younger animals
Prostate and seminal vesicles		
Atrophy	Organ weight decrease of the seminal vesicle and/or prostate is the most sensitive indicator of low testosterone or antiandrogenic activity. It can often be the only evidence of an effect on androgen imbalance and occur in the absence of testicular changes. Organ weight is also more sensitive than histopathologic assessment of atrophy or hypertrophy in these tissues.	The degree of acinar or vesicle distension/ contraction and the secretory content of the prostate and seminal vesicles is variable. Focal areas of atrophic prostatic acini are often present in control animals. Decreased body weight gain/food intake and increased stress result in decreased LH and testosterone, which is reflected by decreases in prostate and seminal vesicle weight. Testes and spermatogenesis are morphologically unaffected by up to 30% decreases in body weight gain (compared with controls).

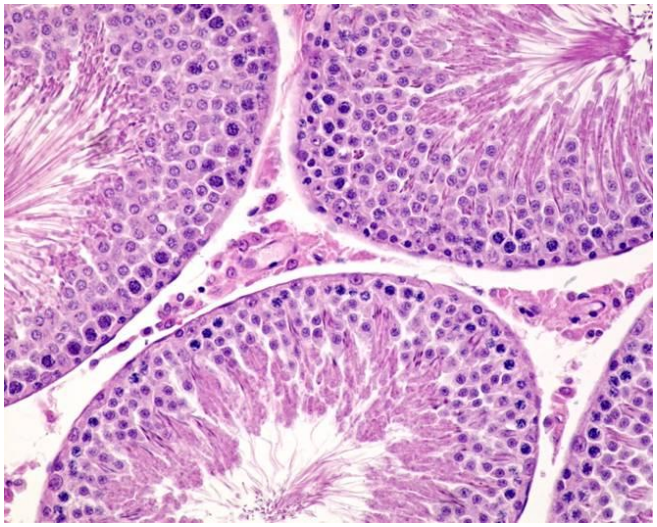
Profile of testicular changes with low testosterone

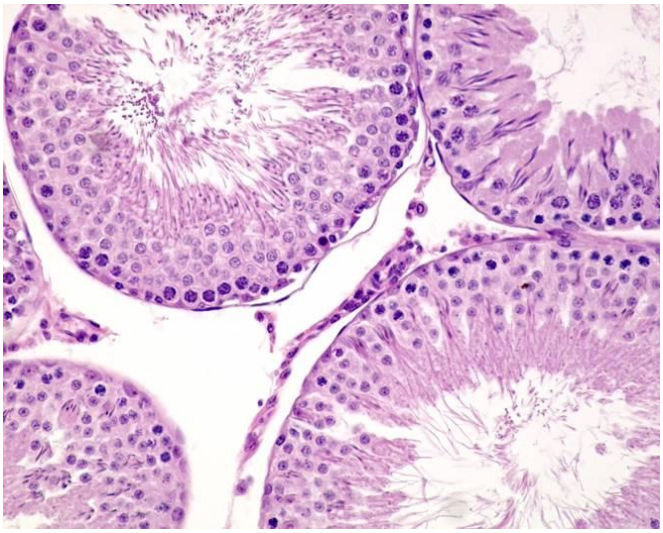


Degeneration of occasional pachytene spermatocytes and round spermatids in stage VII and VIII tubules. This is a very sensitive and early marker of decreased testosterone levels in the testis and is very specific to this mechanism of toxicity. Leydig cell atrophy is also present.

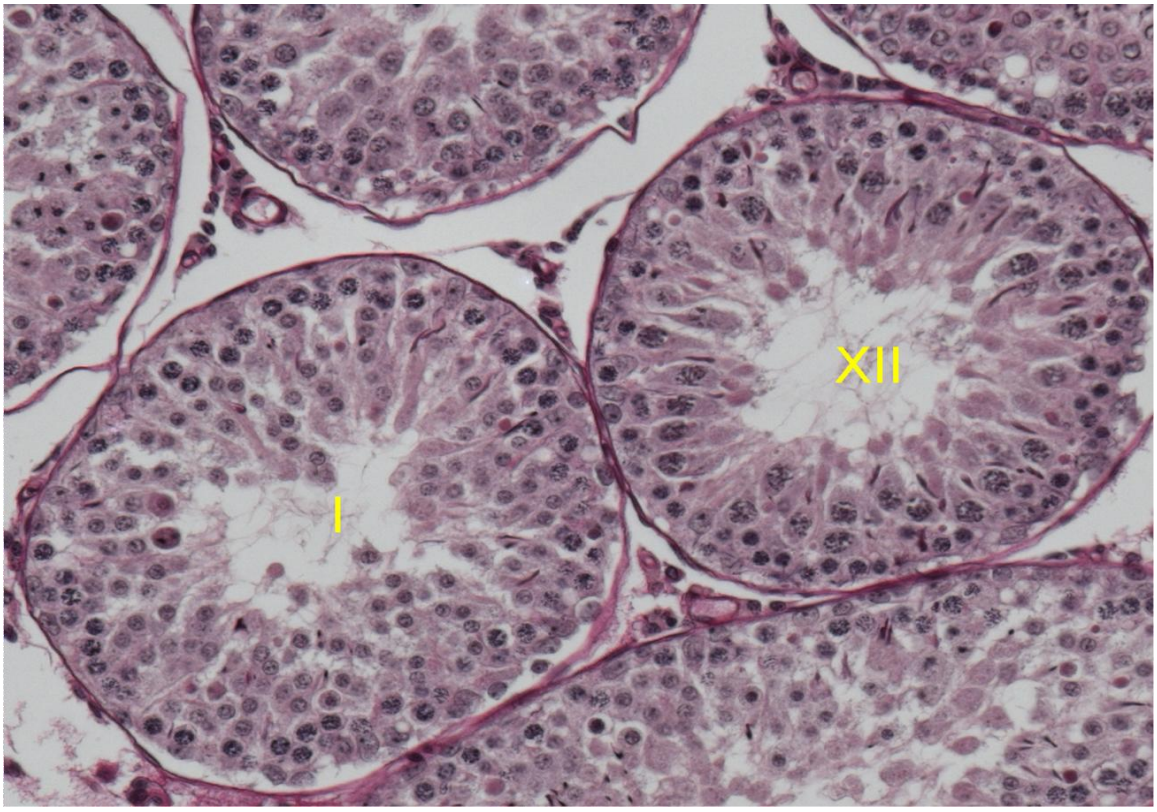


Spermatid retention: this is a very early and sensitive indicator of testosterone depletion. However it can be seen with other mechanisms of toxicity.



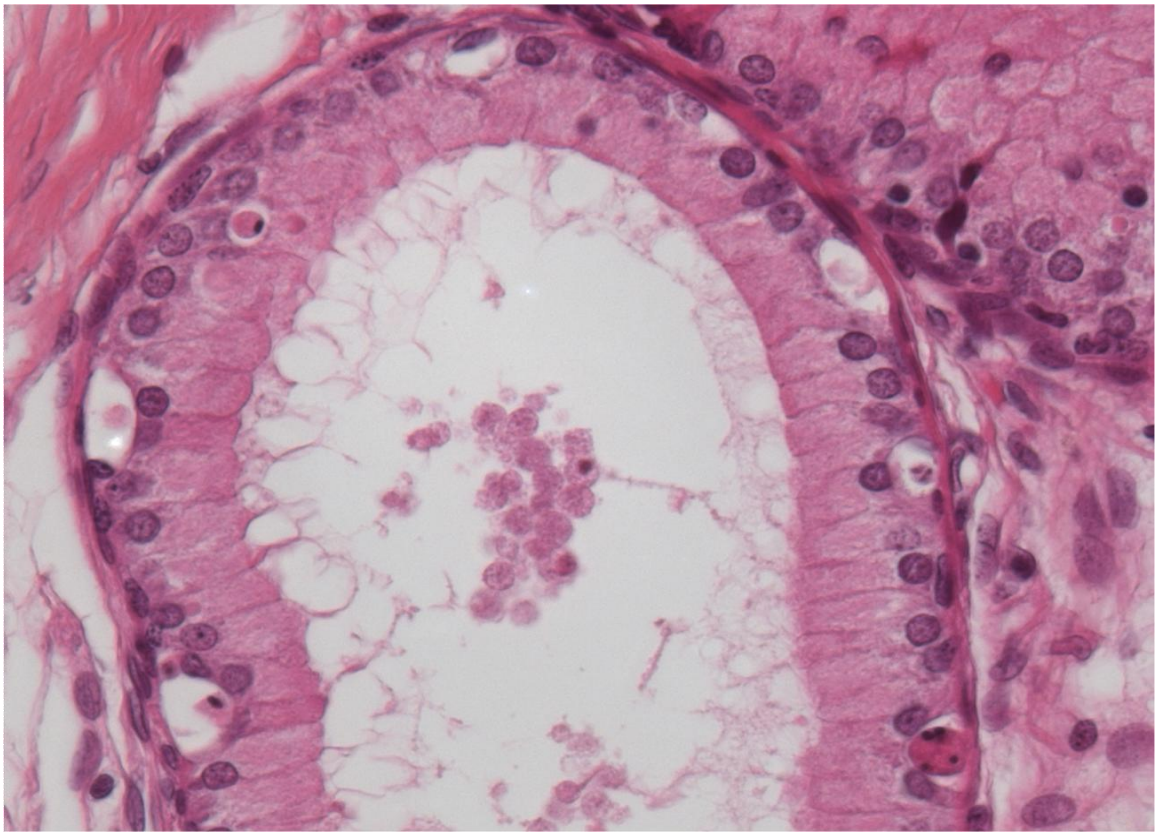


Leydig cell atrophy (right). This is not a very sensitive endpoint but can be identified when steroidogenesis is markedly inhibited.

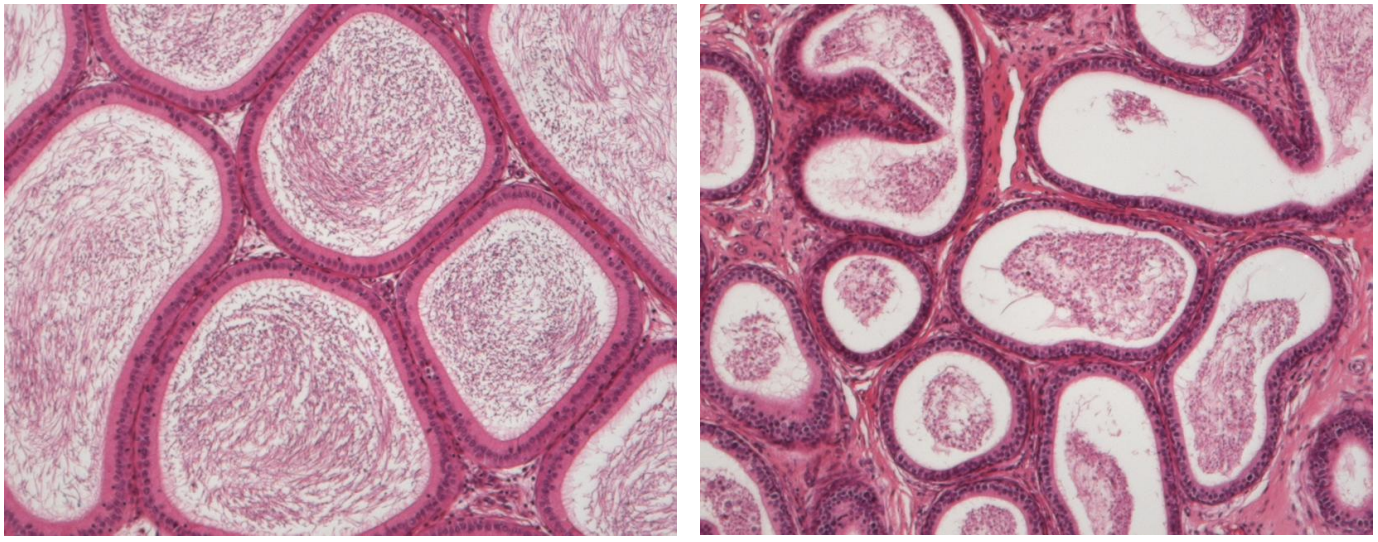


Generalized degeneration and loss of elongating spermatids from all stages of the spermatogenic cycle accompanied by atrophic Leydig cells. This is the end stage lesion of chronic or severe testosterone depletion.

Profile of epididymal changes with low testosterone

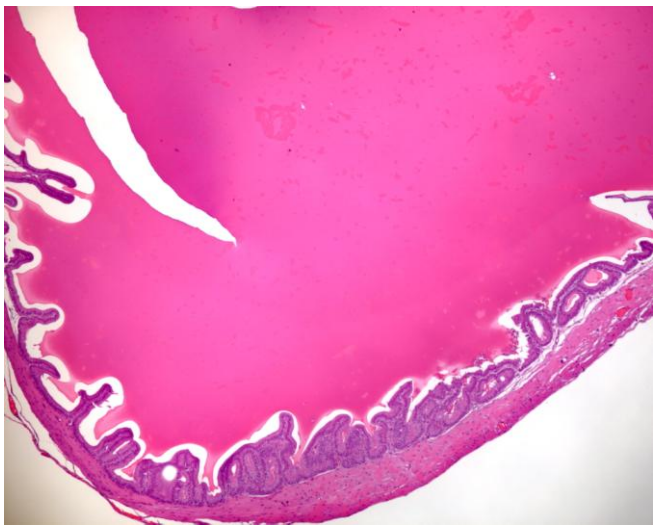


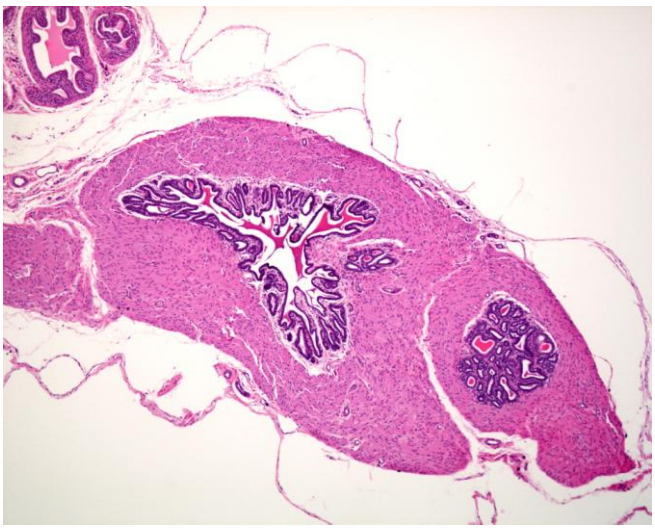
Apoptosis of epithelial cells in a small region of the epididymis in the initial segment or proximal caput.



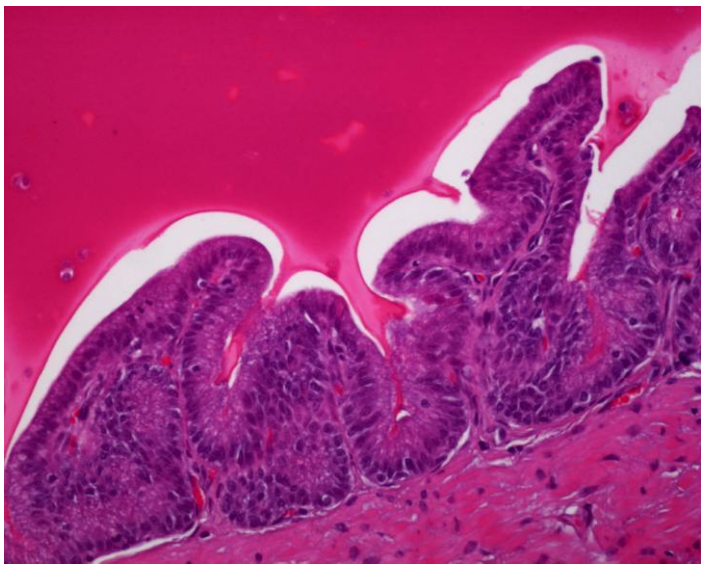
Normal epididymis (left). Ductal atrophy of the epididymis with decreased sperm (right). This is a relatively late effect of low testosterone, which reflects the decreased spermatogenesis and spermiation in the testis.

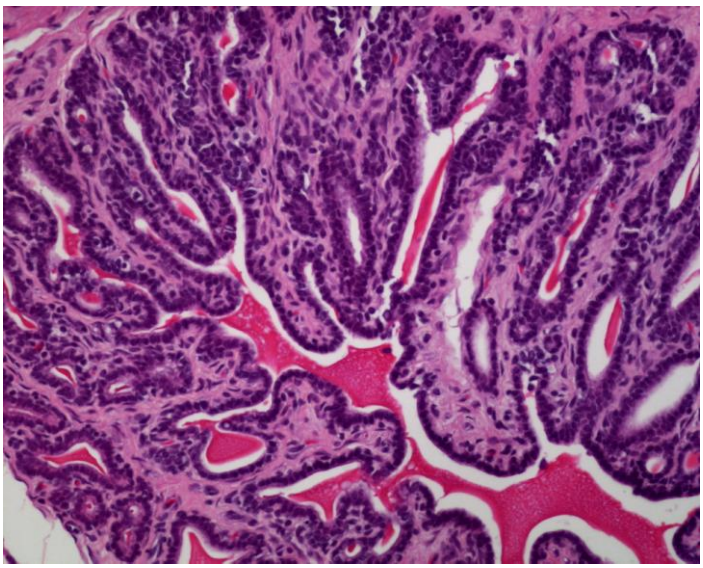
Profile of accessory sex organ changes with low testosterone



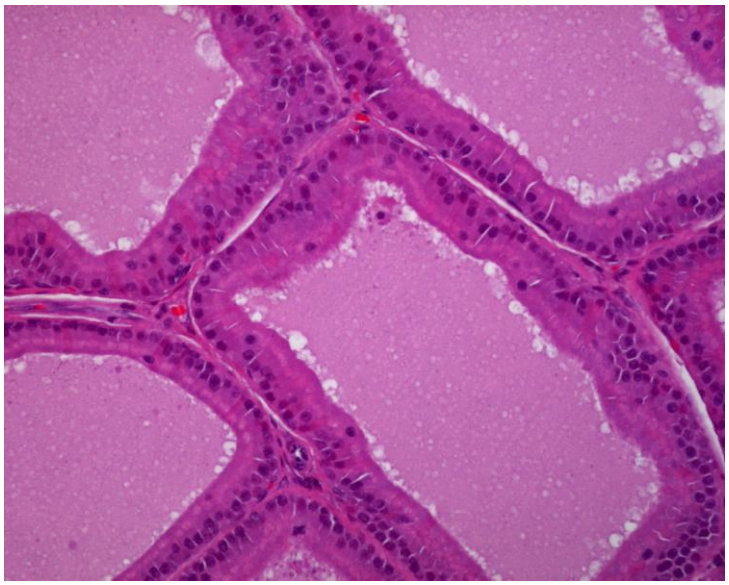


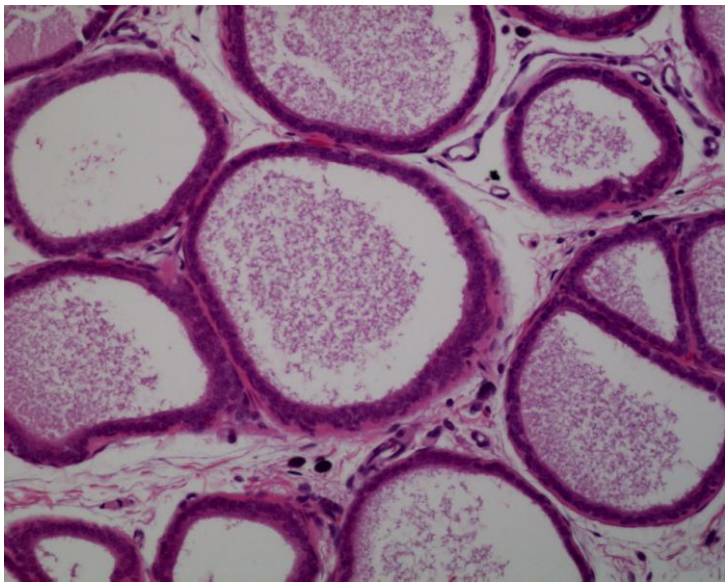
Seminal vesicle contraction and decreased secretion (right). This is only seen with marked reductions in testosterone. Decreased organ weight is more sensitive for lesser reductions.





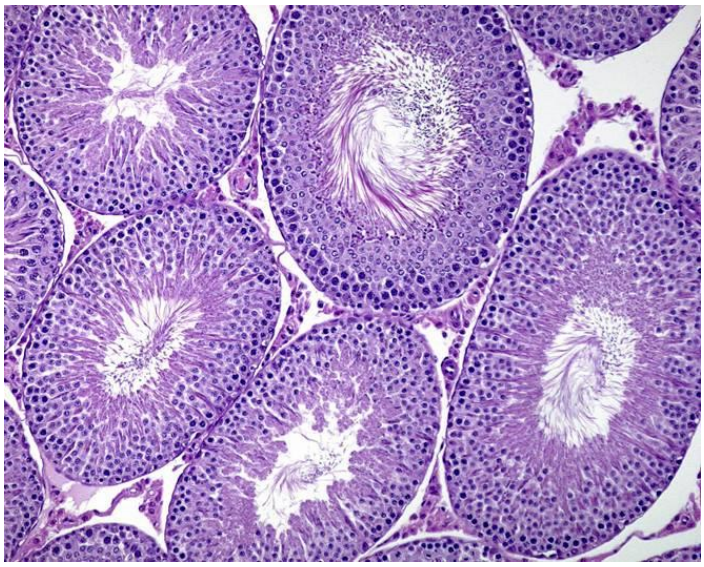
Note decreased epithelial height and loss of apical secretory droplets in the atrophic tissue (right).

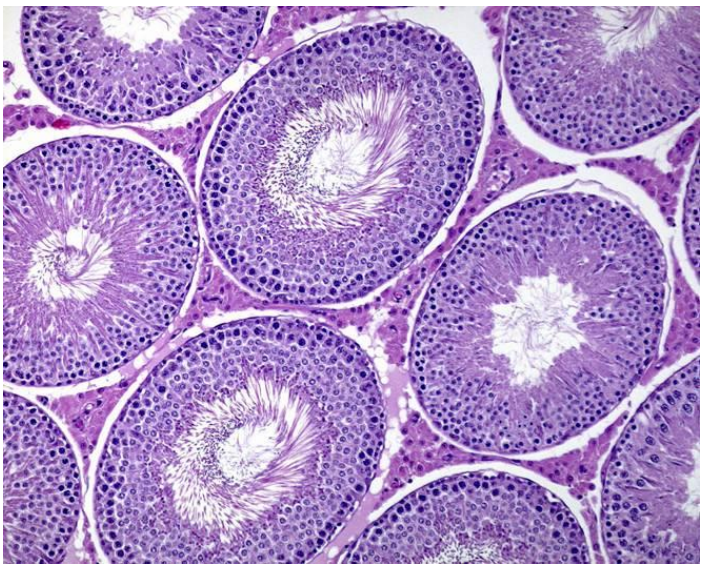




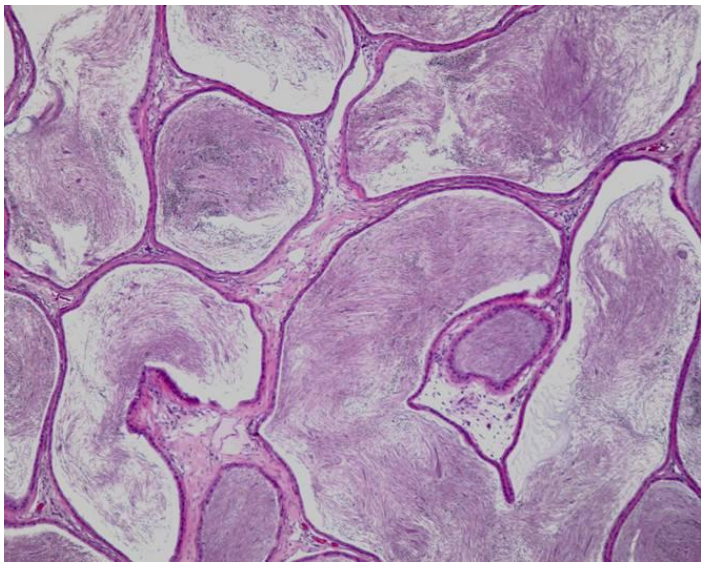
Atrophy of ventral prostate (right). Note loss of secretory droplet from apical cytoplasm in the atrophic acini. This is only seen with marked reductions in testosterone. Decreased organ weight is more sensitive for lesser reductions.

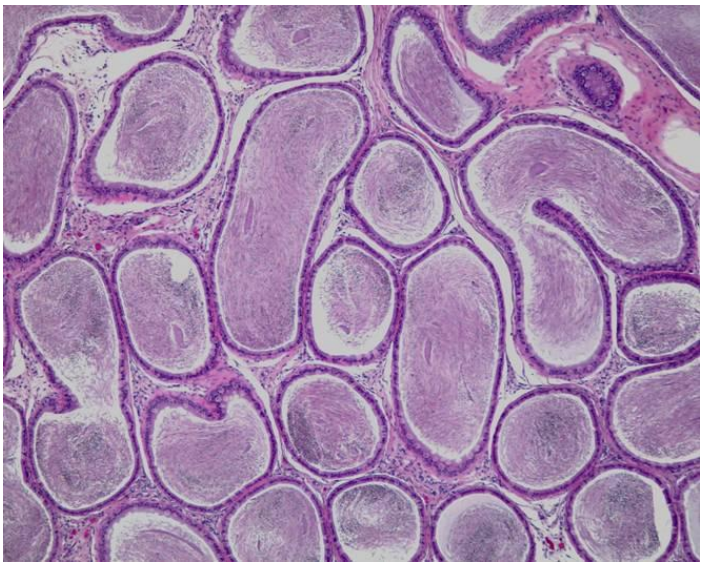
Profile of testicular and epididymal changes with androgen receptor antagonism





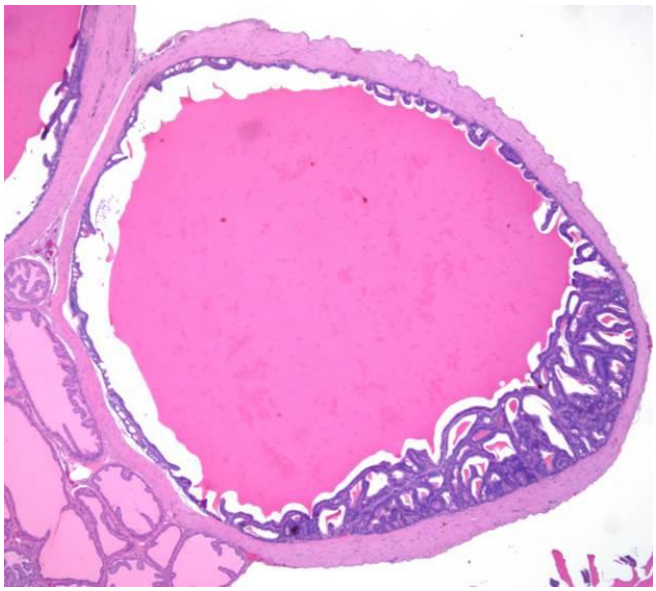
AR antagonism has very little effect on spermatogenesis. AR antagonism acting on the hypothalamus and pituitary results in increased LH which causes hypertrophy/hyperplasia of the Leydig cells (right). Compare with control (left).

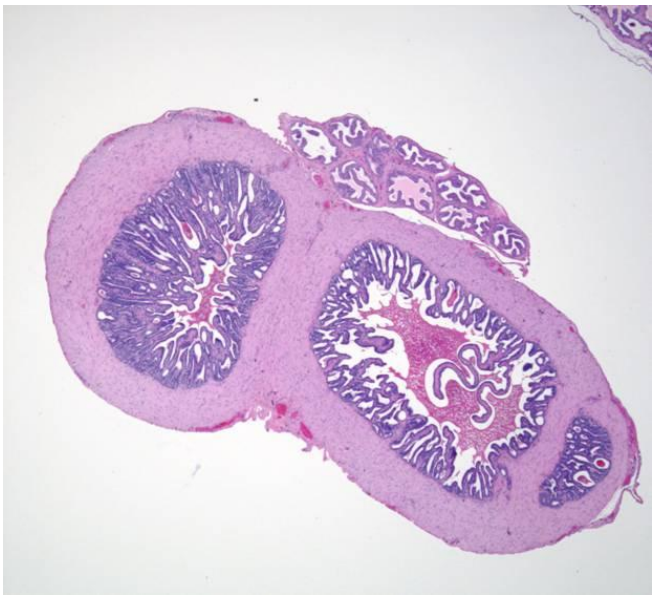




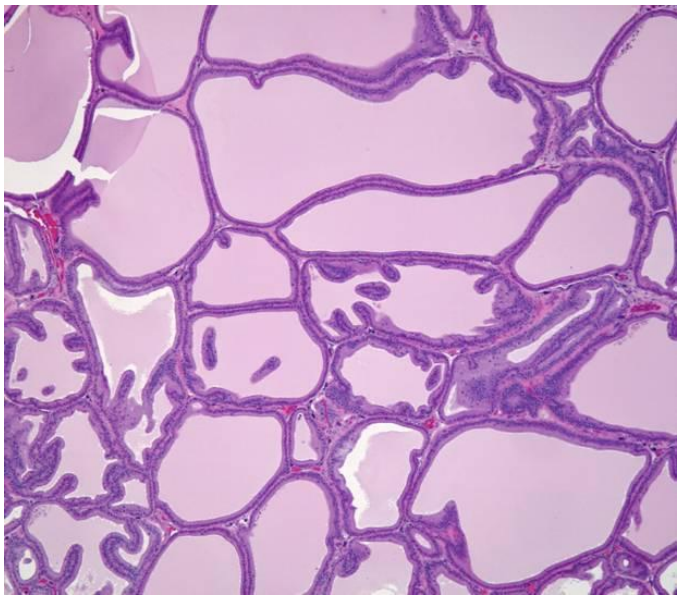
Sperm content of the epididymis is relatively normal but there is slight atrophy which is more easily recognized by decreased epididymal weight. Control (left), AR antagonist (right).

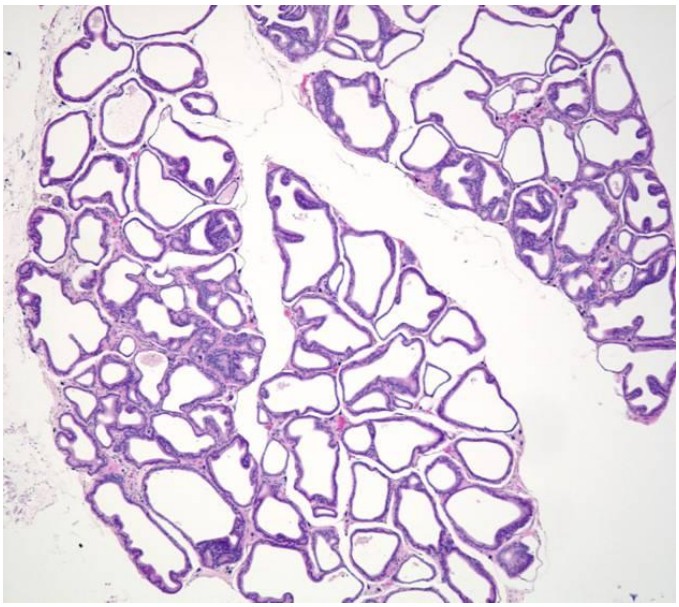
Profile of secondary sex organ changes with androgen receptor antagonism





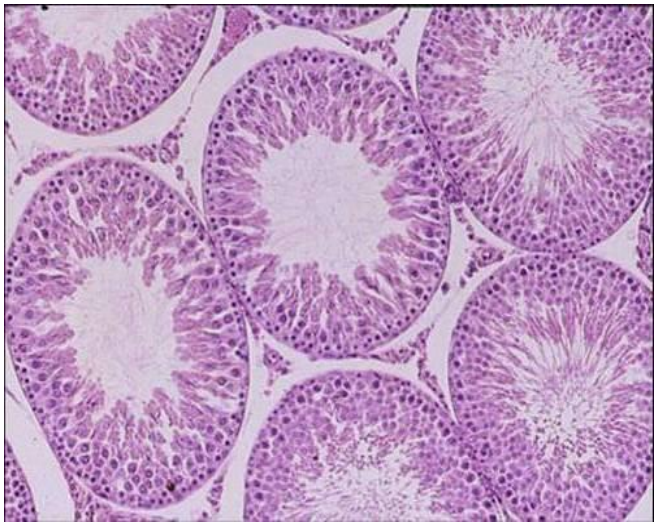
AR antagonism causes marked atrophy of the seminal vesicle and marked reduction in organ weight but with a concomitant increase in serum LH and testosterone levels.

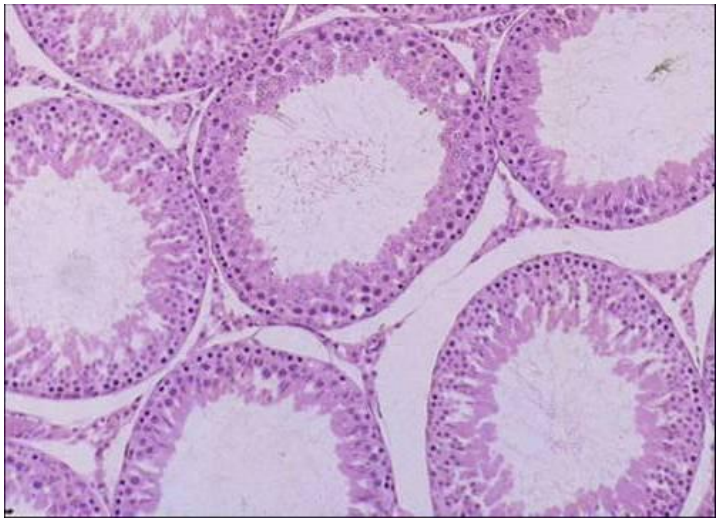




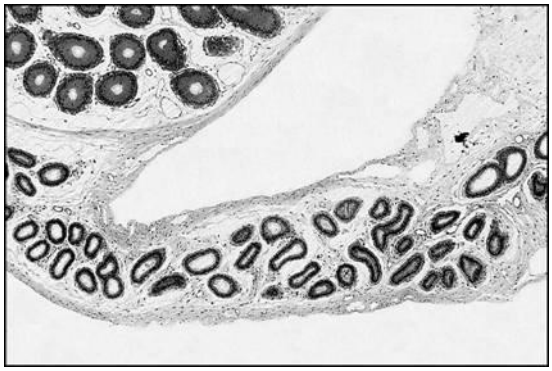
AR antagonism causes marked atrophy of the prostate with decreased organ weight.

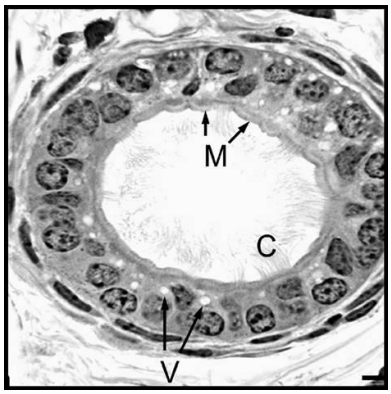
Profile of testicular and efferent duct changes with oestrogen antagonist



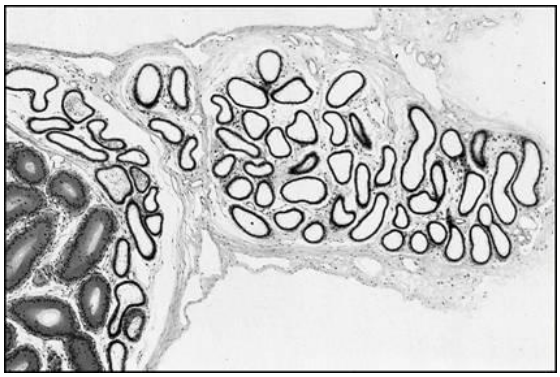


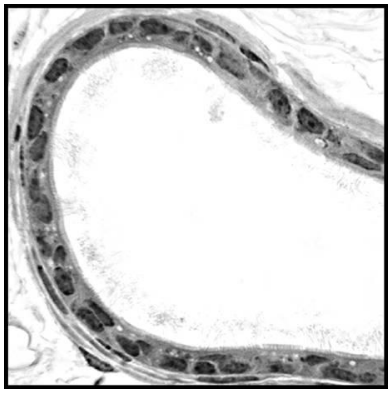
Dilatation of seminiferous tubular lumens (right) which is generally associated with increased testis weight. Control (left).
Below: increased diameter of efferent duct lumens.





Control





ICI 182,780 (antioestrogen)

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