ASSESSMENT OF THE ROLE OF TESTOSTERONE IN THE RAT GONADS: MORPHOMETRICAL AND CYTOCHEMICAL ANALYSIS

A DISSERTATION
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DEPARTMENT OF BIOLOGY

BY

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DENTON, TEXAS
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Nov. 8, 1996
Date

To the Associate Vice President for Research and Dean of the Graduate School:

I am submitting herewith a dissertation written by Gladis A. Shuttlesworth entitled "Assessment of the role of testosterone in the rat gonads: Morphometric and cytochemical analysis." I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

Nathaniel C Mills, Major Professor

We have read this dissertation and recommend its acceptance:

Department Chair

Accepted:

Leslie M Thompson
Associate Vice President for Research and Dean of the Graduate School

To my mother Angela Correa Tello
whose love, faith, and determination
continue to be an inspiration and
a driving force in my life.

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Gladis A. Shuttlesworth
Abstract

ASSESSMENT OF THE ROLE OF TESTOSTERONE IN THE RAT GONADS: MORPHOMETRICAL AND CYTOCHEMICAL ANALYSIS

December 1996

Rat testes are composed of somatic cells, primarily Sertoli (SC), myoid (MC) and Leydig cells (LC), and germinal epithelium which gives rise to spermatozoa. Sertoli, myoid, and Leydig cells have androgen receptors, of which Sertoli and myoid cells are thought to be primarily responsive to testosterone. Adjacent to Sertoli and myoid cells are Leydig cells which are the primary source of testosterone (T). The aim of this study is to evaluate the effects of T on testicular somatic cell morphology and changes in protein synthesis profiles, using prenatally irradiated male rats (devoid of germ cells) that are, thus, Sertoli-cell enriched (SCE).

Mature SCE rats were treated with Leuprolide (Leupr.) a GnRH agonist, for six weeks to prevent release of LH and FSH from the pituitary, thus, indirectly blocking T production from Leydig cells in the absence of LH. Some SCE rats were treated with testosterone propionate (T.P.) or in combination with Leupr. Testis weight (with and without tunica albuginea) of Leupr. treated groups were reduced to 53% of SCE controls. Seminiferous tubule diameter of Leupr. treated animals was reduced to 70% of the control. Neither testicular weight not tubule diameter were maintained by T replacement in Leupr. treated animals. Testosterone levels were reduced to 30% of SCE controls with Leupr. Androgen replacement with or without Leupr. increased T levels 17 and 13 fold respectively. With Leupr., Leupr. + T.P., or T.P. alone there was a two fold decrease of LH concentration as compared to the SCE control. FSH levels of groups treated with Leupr. alone and in combination with T.P. showed 94% and 81% higher and lower values, respectively, when compared to SCE controls.

Leydig cells showed a severe morphological change in the Leupr. treated group and interstitial tissue had an abundance of unidentified cells. Myoid and Sertoli nuclear morphology was unaltered and no statistical difference in SC numbers were observed.

Protein profiles of the testicular tissue were analyzed using SDS-PAGE. A newly synthesized protein (36kDa) was secreted into the medium with Leupr. treatment. This protein was not observed in control, or in T.P. treated animals, but it was found occasionally in the Leupr. + T.P. treated group.

Results obtained in this study appear to indicate a direct effect of Leuprolide in somatic cell morphology and in changes of the protein synthetic profile.

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CHAPTER I

INTRODUCTION

Structural Organization of the Testes

The testes are composed of seminiferous tubules which contain germ cells and somatic cells, and the interstitial tissue of the intertubular space. In rat, the tubules are symmetrically folded in a conical orientation with each end of the tube system opening to the rete testis for collection of mature spermatozoa. The seminiferous epithelium is comprised of the somatic Sertoli cells and the germinal cells which produce the maturing haploid gametes. The interstitial tissue is composed of blood and lymphatic vessels, nerve fibers, macrophages, connective tissue cells, the lamina propria of the seminiferous tubules or myoid cells (modified smooth muscle cells) and Leydig cells, the androgen secreting cells of the testes. The seminiferous epithelium is comprised of the somatic Sertoli cells and the germinal cells which produce the maturing haploid gametes.

Spermatogenesis

Spermatogenesis is the development and maturation of male gametes from spermatogonia (Figure 1). The whole process in the rat takes approximately 48 days and is divided into 14 cellular association which cycle every 12 days.

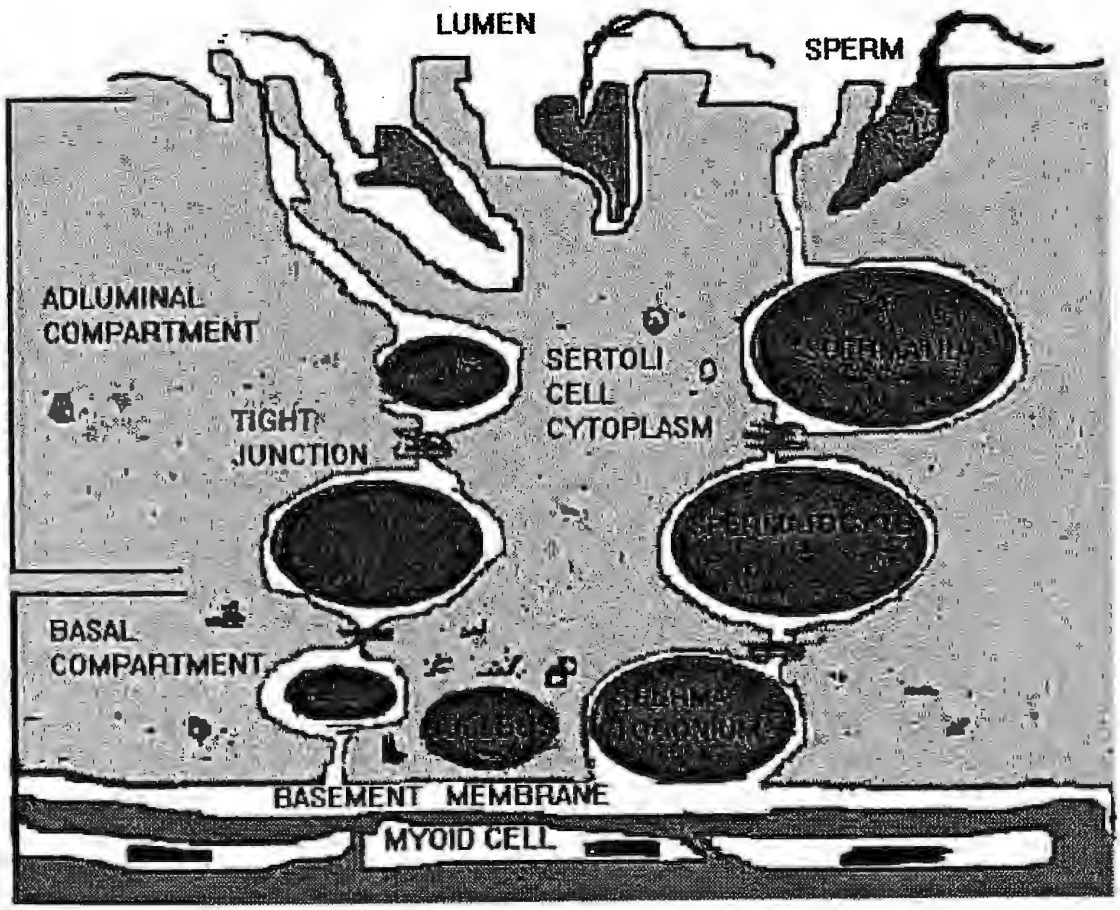


Figure 1. Events of Spermatogenesis

Spermatogenesis is the development and maturation of male gametes from spermatogonia which become spermatocytes and spermatids through a series of mitotic and meiotic divisions and culminating in the formation of mature spermatozoa. The events of spermatogenesis take place in the microenvironments created by plasma membrane indentations of adjacent Sertoli cells.

The cycle of the seminiferous epithelium has been defined as that series of changes occurring in a given area of the seminiferous epithelium between two successive appearances of the same cellular association. Leblond and Clermont (1952) have described 14 different cellular associations in the seminiferous epithelium of the rat due to timed mitotic and meiotic divisions of cell clusters linked by cytoplasmic bridges. Mitotic division increases spermatogonial numbers, that divide meiotically into spermatids which undergo morphogenesis (spermiogenesis) resulting in the formation of haploid spermatozoa. At each stage of the cycle, germ cells are intimately associated with Sertoli cells. The more primitive cells (spermatogonia) are located in the basal compartment between the myoid cells and are partially surrounded by the Sertoli cell membrane and cytoplasm. After completion of DNA replication, meiotic cells move into the protective environment provided by the Sertoli cell's cytoplasm. The synchronous progression of the spermatogenic process suggests that a mechanism may exist to coordinate genetic, biochemical and morphological events. Numerous enzymes, DNA-binding proteins, and structural proteins have been shown to be transiently expressed at specific intervals of spermatogenesis (Hecht, 1990).

Testicular Blood Flow

Testicular blood flow is the main pathway for the transport of nutrients, regulatory hormones, and secretory products to and from the testis. As in all other organs, control of blood flow is therefore important, and may be particularly critical for the testis since the concentration of oxygen in the

seminiferous tubules is very low. A strong positive correlation exists between testicular blood flow and the output of testosterone into the spermatic vein (Bergh and Damber, 1993). Consequently, testosterone secretion may be influenced rapidly by changes in blood flow (provided that arterial blood contains a constant concentration of luteinizing hormone (LH) and other factors that directly influence steroidogenesis in the Leydig cells).

Tight Junctions

Tight junctions develop between Sertoli cell plasma membranes, resulting in the blood-testis barrier which separates the interstitial space from the adluminal and the luminal compartments where meiotic cells mature, and immature spermatozoa are transported through the lumen for storage and maturation in the epididymis. Tight junctions maintain a polarity of secretory products from Sertoli cells. Some products are directed apically toward the germ cells in the adluminal compartment and/or the lumen of seminiferous tubules; other secretory products are directed basally toward the interstitium (Setchell and Waites, 1975). In mammals as well as in birds, reptiles, fish, amphibians, insects and nematodes, the establishment of inter-Sertoli cell tight junctions during postnatal life triggers maturation of Sertoli cells and is the prerequisite for the normal development of meiosis and spermiogenesis (Jegou, 1992).

Sertoli Cells

Sertoli cells are a primary constituent of the seminiferous epithelium having first been described as phagocytic cells (Kerr and de Kretser, 1974;

Pineau et al., 1991). The phagocytic process involves the elimination of degenerating germ cells that die during the spermatogenic process. Sertoli cells also secrete seminiferous tubule fluid, peptides, steroids, and proteins. Some of these proteins may influence germ cell maturation. According to Bardin et al. (1988) more than 100 proteins may be secreted and most of these proteins remain to be characterized and identified (Jegou, 1992). Some of these proteins are testes specific and others are homologous to serum proteins (Wright et al., 1981). During puberty, Sertoli cells cease division and enter a maturation phase which results thereafter in a constant number of Sertoli cells per unit length of the seminiferous tubule. This number is constant at all stages of the cycle of the seminiferous epithelium after that age at about 16 x 10^6 cells/g testis (Mori and Christensen, 1980).

Myoid Cells

Myoid cells (peritubular cells), and Sertoli cells comprise a morphological and functional unit that forms the cytoarchitectural scaffolding of the seminiferous tubule. Both cooperate in the production and formation of a complex extracellular matrix, or basement membrane of the tubule (Skinner et al., 1985). Myoid cells are mesenchymal. They surround the Sertoli cells and form the exterior wall of the seminiferous tubule. They synthesize and secrete a paracrine factor termed P-Mod-S (protein that modifies Sertoli cells), which can modulate the function, differentiation, and total protein secretion of Sertoli cells and may indirectly influence the process of spermatogenesis (Skinner and Fritz, 1986).

Leydig Cells

Leydig cells, located in the interstitial space between seminiferous tubules are also derived from mesenchymal precursor cells which then proliferate during puberty (Hardy et al., 1989). The development of precursor cells into steroid-producing cells is a complex process that involves a plethora of morphological and physiological changes (Teerds et al., 1989a; Shan and Hardy, 1992). Leydig cells normally contain round or oval nuclei with smooth nuclear and cellular margins. In the whole testis, 82.4% of the volume is occupied by the seminiferous tubules, 15.7% by the interstitial tissue, and 1.9% by the tunica albuginea. Leydig cells constitute 2.7% of testicular volume (Mori and Christensen, 1980), which means that each cubic centimeter (~1 g) of rat testis contains about 22 million of them. A Leydig cell secretes an average of ~0.4 pg of testosterone/day or 10,600 molecules of testosterone /second (Mori and Christensen, 1980). Therefore, the total Leydig cell population in 1g of rat testis produces 6.7 ng of testosterone/min *in vivo* (Free and Tillson, 1973). The biosynthesis of testosterone is catalyzed by enzymes located predominantly in the membranes of the smooth endoplasmic reticulum (SER) and in adjacent cytoplasm.

Pituitary Hormones

The pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), act independently but synergistically and are thought to play an important role in spermatogenesis (Kerr et al.,1992). LH exerts effects on the spermatogenic process by modulating the secretion of testosterone (T) by

Leydig cells, whereas FSH acts directly on Sertoli cells. The synthesis and release of LH and FSH from the pituitary are, in turn, regulated by the hypothalamic peptide gonadotropin releasing hormone (GnRH) and are subject to feedback regulation by testosterone and estrogen (Figure 2).

There is still a debate over the precise level of testosterone required to maintain quantitatively normal spermatogenesis. The mechanism(s) by which testosterone exerts its supportive effect on spermatogenesis is not known. However, it must occur via the Sertoli cells and peritubular cells, since these are the the only cells in the seminiferous tubules that express androgen receptors.

Early experimental rat models provided evidence that T alone is capable of maintaining (Awoniyi et al., 1992) or restoring (Awoniyi, 1989) spermatid number in the rat in which LH, T, and FSH are suppressed by active immunization against GnRH, suggesting that FSH has no direct role in the regulation of spermatogenesis. However, recent experiments have demonstrated that spermatogenesis is not quantitatively restored in GnRH-immunized rats which received replacement amounts of testosterone (Sharpe, 1994). Similarly, in hypophysectomized rats, spermatogenesis was not quantitatively maintained or restored by administration of high doses of testosterone (Santulli et al., 1990). Therefore, quantitatively normal spermatogenesis may not depend on the high intratesticular androgen levels present under normal conditions. The physiological importance of the high testicular androgen concentrations still remains unknown. However, the testis

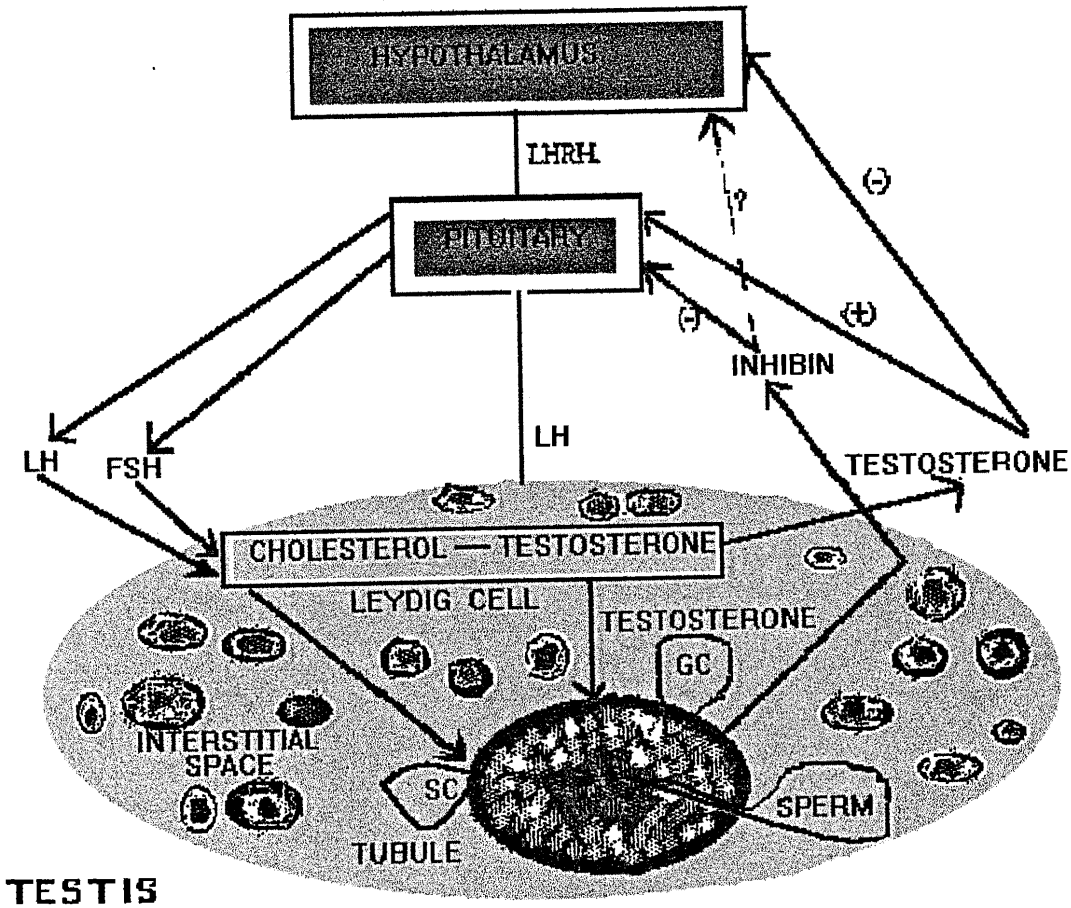


Figure 2. The Hypothalamic-Hypophyseal-Gonadal Axis

Luteinizing hormone releasing hormone (LHRH) from the hypothalamus triggers the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. LH, in turn, triggers the synthesis of testosterone by Leydig cells located in the interstitial space in the testis. Feedback, both positive and negative, of testosterone and inhibin (produced by Sertoli cells (SC)) at the pituitary and hypothalamus is shown.

forms the reservoir from which the peripheral blood stream and target organs are provided with testosterone. Considering the relatively low blood flow through the testis, the reservoir must contain a massive testosterone concentration (maintained by continuous production) to insure adequate supply to the periphery. Thus, the intratesticular concentrations might only be high because testosterone has an endocrine function in addition to the local paracrine function (Weinbauer and Nieschlag, 1993).

Most of the evidence for the effects of testosterone on testicular cell populations was obtained from isolated cultured cells and the effects were less pronounced than expected (Verhoeven et al., 1995). *In vitro* studies performed by Sharpe et al. (1992) demonstrated that testosterone regulates both the overall level of protein secretion and the secretion of several specific proteins by seminiferous tubules at stages VI-VIII of the cycle of the seminiferous epithelium.

The effect of testosterone on somatic cell morphology and protein synthesis in irradiated male rats *in vivo*, has been a topic of few studies. To date, the work of Verhoeven et al. (1995) appears to be the only study appearing in the literature that investigated the androgen effect on somatic cells using prenatally irradiated rats treated with GnRH agonist for 3 weeks. His work has shown that testosterone does not have a major effect on the synthesis of a specific protein but causes only an overall increase in protein and RNA synthesis. This result suggests that testosterone is unable to regulate specific genes in somatic cells.

Paracrine Regulation of Testicular Function

A consensus among investigators is that intercellular communication also occurs within and between testicular compartments. The paracrine regulation of testicular function is synergistically modulated by Sertoli and myoid cells by several growth factors (Niederberger et al., 1993). The role of Sertoli cells in relationship to Leydig cells was demonstrated by induction of damage to spermatogenesis (i.e. loss of germ cells) resulting in hypertrophy of the neighboring Leydig cells. This response occurs regardless of whether the damage is induced by X- irradiation (Rich et al., 1979) cryptorchidism (Risbridger et al., 1981a), vitamin A deprivation (Rich et al., 1979), or by chemicals (Aoki and Fawcett, 1978). Likewise, Sertoli and germ cells interactions mediated by soluble factors were demonstrated by Onoda and Djakiew (1990). Cell to cell interaction can modify the response of a cell to testosterone.

Statement of the Problem

Taking all these studies into consideration, the present investigation was designed to evaluate the effects of androgen on somatic cell morphology and protein synthesis in irradiated male rats *in vivo*. To enhance the androgenic effects on the testes, two rat model systems were used.

The first involved elimination of germ cells. Unlike Sertoli cells (Sanborn et al., 1977) and myoid cells (Nakla et al., 1984) which contain androgen receptors, the germ cells which comprise 80% of normal, mature, gonadal cellular populations, are not reported to contain androgen receptors

(Grootehoed et al., 1977). Therefore, using *gamma*- irradiation *in utero*, the large protein background created by the presence of germ cells in the normal testis can be eliminated, and consequently, the evaluation of androgen action can focus on androgen receptor- containing, somatic cell populations (mostly Sertoli cells, myoid cells, and Leydig cells).

The second model was produced by increasing levels of GnRH to supersede GnRH pulsatile release from the hypothalamus, thereby decreasing LH and FSH secretion and hence suppressing testicular steroidogenesis. The androgen levels in the gonads are normally 10 to 20 times greater than in peripheral circulation. In order to evaluate the action of androgen on the somatic cells, it was necessary to reduce endogenous androgens in the mature gonad. Exogenous androgen can be administered to compare morphology and protein profile changes with and without androgens.

The withdrawal of testosterone was accomplished by administering a small synthetic peptide, an agonist of gonadotropin releasing hormone (GnRH or Leuprolide, Tap Pharmaceutical) which suppresses the production of LH and FSH from the pituitary. If GnRH is made available to the anterior pituitary in a continuous drip or a depot injection, an initial surge (up-regulation) in the levels of LH and FSH will be followed by a sharp decline in concentration (down-regulation). LH and FSH levels then remain depressed until GnRH is available on an intermittent (pulsatile) schedule. This activity is the result of the continuous availability of releasing factors that saturate the receptor sites of the neurohormones in the anterior pituitary gland and, in turn, lead to

desensitization and down-regulation. The effects are also partly attributable to gonadotropic hormone receptor depletion. The half life of endogenous GnRH has been estimated as approximately 2 to 4 minutes, but Leuprolide has a half life of approximately 3 hours and has a biological potency up to 300 fold higher than that of endogenous GnRH (Garcia, 1993). It is possible that this treatment has a less detrimental effect than hypophysectomy, since the pituitary is still present (Roberts and Zirkin, 1991).

Clinically, Leuprolide has been recommended for the treatment of prostatic carcinoma, endometriosis, uterine fibroids, and breast cancer (Rao et al., 1993). It is also currently used for treatment of precocious puberty, and leiomyomas (Fekete et al., 1989). However, the GnRH effects on testicular function have not been evaluated in detail. The GnRH agonist effect on testes is mediated via membrane receptors and only Leydig cells appear to possess such receptors. As a result of agonist treatment, Leydig cells which require LH to maintain their structure and functional activity (Ewing and Zirkin, 1983), will atrophy (Christensen and Gillim, 1969) and may be unable to produce testosterone (Miller, 1992).

Significance of the Project

Data obtained by utilizing these two models, will provide new insights concerning the influence of testosterone on the morphology of testicular somatic cells. In addition, the qualitative and relative quantitative assessment of a variety of testicular parameters, in particular the somatic cells, by means of

histological analysis, will yield relevant information about androgen effects and the mechanism of hormone action.

When morphology is correlated with alterations in the protein synthetic profile an understanding of hormonal regulation during spermatogenesis can be developed. For example, one of the objectives of the present study is to examine the changes in mature rats that occur in the absence of LH and FSH eliminated by an GnRH agonist and/or following administration of testosterone. This may enable a determination of: (a) which somatic cell is more sensitive to the elimination of these hormones; (b) whether the administration of testosterone will maintain somatic cell morphology; (c) whether factors other than testosterone are necessary to maintain normal morphology.

Of equal importance in this project is to answer the following question: Are there qualitative changes in the total secretory and/ or cellular protein profiles in each treated group? This will enable an evaluation of the effects of testosterone in the testis, which might contribute to a better understanding of the process of spermatogenesis. For instance, changes in the protein profile in the treated groups due to decreases or increases in specific proteins show whether a particular protein is androgen regulated. The attempt to identify relevant proteins by comparing their characteristics with those known to be synthesized or secreted by somatic cells in the testis could also lead to an understanding of their possible functional significance in the spermatogenic process.

Protein profile changes obtained in this study may lead to future studies on the characterization of the most relevant proteins, which could be of major importance in the development of novel diagnostics and to prognosis and therapeutic means for the treatment of prostatic cancers and other endocrine disorders. Furthermore, understanding the biochemical changes of the somatic cells under the treatments used in this study may enhance our knowledge of the etiology and pathology of different diseases in the testis and in its accessory sex organs. This, in turn, could potentially lead to improvement in the treatment approaches to these disorders, since biochemical changes of somatic cells in the testis *in vivo* have been the topic of only a few studies.

These findings may also contribute to further understanding of the reproductive process relevant to birth control in males and females, since LHRH and LH are very important regulatory proteins in this regard, leading to additional means of improving fertility methods in both genders. Thus, Leydig cells, which synthesize testosterone in the male, have a female counterpart known as theca cells which synthesize testosterone and provide the substrate for granulosa cells to synthesize estrogen. LH acts in females to induce ovulation and maintain the secretory functions of the corpus luteum. In males, LH acts by stimulating the Leydig cells of the testes to produce testosterone. Therefore, both organs could respond to the presence or absence of androgen in a similar fashion.

Since Leuprolide is currently used to treat prostatic cancer, endometriosis and fibroid tumors, and the half life of this agonist is longer than

that of GnRH, these results may facilitate further understanding of gonadotropin action in the testis, ovary, and other sex steroid-dependent organs. Understanding the effect and extent of gonadotropin action may lead to changes in hormone treatment currently used in the medical field.

Ultimately, the results would provide a framework for future investigations at the molecular level into changes in testicular gene expression upon treatment with a GnRH agonist for 6 weeks or longer.

CHAPTER II

MATERIALS AND METHODS

Materials

Leuprolide, [leuprorelin; D-Leu^{6}-(des-Gly^{10}-NH_2)-LHRH ethylamide acetate] in the microsphere injectable form was a gift from Tap Pharmaceuticals Inc., North Chicago, IL. Fixatives, and Histochoice were purchased from Amresco, paraformaldehyde was from Fisher Scientific, Dextran-T40 was from Pharmacia.

Animals Models

Female rats (Strain F344) were irradiated on the 20th day of pregnancy with 180 rads from a Cobalt-60 source (Mills, 1990) to obtain male offspring with testes free of germ cells but enriched with Sertoli cells. Between 20-25 days after birth, irradiated males were weaned and housed in groups of two per cage under conditions of controlled temperature and humidity and given free access to rat chow and water. The protocol used was reviewed and approved by the Animal Care and Use Committee of Texas Woman's University (ACUC). Animals were grown to maturity in the TWU vivarium.

Four groups of 6 animals between 60 and 120 days of age were experimentally treated as follows:

(1) The first group of animals (n=6) was used as controls to show the status of the testis without treatment and they were only administered vehicles for each treatment: One injection was given subcutaneously, of a comparable volume of vehicle and microspheres without the GnRH agonist at initiation of the experiment and at 22 days. Sesame seed oil (SSO; 0.1 ml) was given subcutaneously at 3 day intervals for the duration of the 6 weeks which was the regimen for GnRH treatment.

(2) The second group of animals (n=6) was treated with 100 μg/kg body wt/day of Leuprolide in microspheres as a depot injection. A rat of 250 g body weight was injected subcutaneously with a single dose of about 700 μg/0.3 ml diluent for 4 weeks utilization. A second dose of 700 μg/ 0.3 ml of diluent was injected subcutaneously at 22 days. The average daily release rate of the Leuprolide was 25 μg of the dosage administration over a period of 6 weeks. Animals also received 0.1 ml of sesame seed oil every third day for the duration of the experiment.

(3) The third group of rats (n=6) was injected subcutaneously with testosterone propionate (T.P.) dissolved in SSO, at 10 mg/kg body weight (approximately 2.5 mg T.P. in 0.1 ml of SSO) at 3 day intervals for 6 weeks to mimic the androgen levels within the gonads. One injection, of 0.3 ml of vehicle and microspheres without the GnRH agonist was given subcutaneously at the initiation of the experiment and at 22 days of the treatment.

(4) The fourth group of animals (n=6) was injected subcutaneously with 700 μg/0.3 ml diluent of Leuprolide in microspheres or with 100 μg/kg body wt/day. This dose was repeated on day 22 of the treatment. In addition, each animal of this group was injected subcutaneously with 2.5 mg T.P. in 0.1 ml of SSO or 10 mg/kg body weight at 3 day intervals for the 6 weeks to replace the androgens lost from the Leuprolide treatment.

Tissue Collection

Animals were killed by decapitation, and blood samples (serum) were collected and stored at -20 ^{o}C for analysis. The testes were removed, dissected free of fat and connective tissue, using jeweler's forceps and then the tissue was immediately rinsed in 10 ml cold phosphate buffered saline, pH 7.4 (PBS; 130 mM NaCl; 3 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4). Then after blotting excess moisture, the intact testes were weighed individually. The tunica albuginea was removed and seminiferous tubules and connective tissue were weighed.

Fixation of Tissue

For histological and morphometric analysis of tissues a small piece of one testis prior to decapsulation was immediately placed in a glass vial (already labeled for each treatment) containing 5 ml of fixative (0.6X Histochoice, 9% paraformaldehyde, 3% dextran, 10% alcohol, pH 3.2). The

tissue was maintained in the fixative for 4 or 5 h at room temperature then it was sliced into 1-2 mm thick segments using a single edge razor blade. Tissue was then placed in the same fixative and left overnight at 4 °C.

Tissue Dehydration and Embedding

After overnight fixation at 4 °C, testis tissue was dehydrated in the cold with ascending concentrations of ethanol (70%, 90%, and 99% for 2 h each) following by an additional 2 h in 100% ethanol at room temperature. While the tissue was dehydrated, several pieces of polyester wax (BDH Ltd Poole, UK) was cut with a razor blade and placed into a flask. The wax was melted either by putting the flask in an oven at 40 °C or bathing the flask with hot running tap water. The testis was then equilibrated in a mixture of 50% ethanol plus 50% melted polyester wax for 4 h at 40 °C, followed by infiltration with 90% polyester wax + 10% ethanol for 2 h at 40 °C. Each tissue sample was transferred to a stainless steel dish (previously warmed at 40 °C) filled with the mixture of 90% melted polyester wax + 10% ethanol. Then plastic block molds were placed on top of the stainless steel molds and the composite stainless steel dish and plastic mold were immediately placed on ice. Labels were marked in pencil and placed in the top of the plastic mold before the wax solidified. Once the blocks became solidified, they were stored at 4 °C.

Coating Slides for Tissue Sectioning and Collection

Glass slides were cleaned with soapy water (one by one) and rinsed several times with tap water and distilled water respectively. Then, slides were placed in a solution that contained 0.2 N HCl in 95% ethanol for 20 min and rinsed again one by one with tap water and distilled water. The slides were set on end and dried at about 60 °C or at room temperature overnight. After cooling, each slide was subbed with a chromated solution that contained: 0.5% of bacteriological grade bovine gelatin (EM Science), 0.5% of Hipure liquid gelatin (Northland Products) and 0.1% of chrome alum ($CrK(SO_4)_2 \cdot 12H_2O$. The bovine gelatin was dissolved in 80 ml of warmed distilled water which was mixed with liquid gelatin after cooling. The chrome alum was dissolved in 20 ml of distilled water which was added to the gelatin mixture (Shuttlesworth and Mills, 1995). This subbing solution was used to sub the slides individually and it was applied only on the side of the slide previously marked with a pencil. Subbed slides were then set on one end in a vertical position in plastic racks and dried at room temperature or at 60 °C overnight. After drying the slides were returned to the original box for storage at room temperature and used within the following 3 months.

Tissue Sectioning

Using a rotatory microtome AO 820 (American Optical Corporation) placed in a cold room (4 °C) to maintain polyester wax firmness, sections of

7 µm thickness were cut. Ribbon type polyester wax sections were floated onto a 32 °C water bath and allowed to spread before collection on coated slides. Slides were left to dry overnight at room temperature, labeled and stored in slide boxes at 4 °C.

Tissue Staining

Sections were dewaxed and rehydrated in 100% ethanol, air dried for 15 min, again treated with 100% ethanol and air dried for 15 min, followed with 90% ethanol twice for 10 min each and twice in 70% ethanol for 10 min each.

For morphometric studies, histology was stained with Ehrlich's hematoxylin to identify nuclear detail and counterstained with eosin for cytoplasmic observation. Ehrlich's hematoxylin is prepared as a nuclear stain by mixing 1% ammonia alum $Al_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$ and 0.65% of hematoxylin in a solution containing 33.3% ethanol, 33.3% glycerol, and 33.3% distilled water. Sections were placed in 200 ml of Ehrlich's hematoxylin stain for 10 min. Followed by two or three washes with tap water or until no blue color was observed in the water. Then, tissue was immediately placed in 200 ml of eosin stain (0.1% eosin Y, 0.5% glacial acetic acid, and 1 L of 70% ethanol) for 5 min, washed in tap water until no orange color was observed in the water. The tissue was quickly dehydrated using increasing concentrations of ethanol, 70%, 90%, 100% (twice) for two or three min. Stained slides were

transferred to 1:1 alcohol/xylene for 5 min, to xylene for 10 to 15 min and mounted with permount.

Sections were also stained with periodic acid Schiff stain (PAS) to distinguish macrophages from Leydig cells since macrophages are PAS positive (+) and counterstained with Ehrlich's hematoxylin. After dewaxing and rehydration, sections were washed gently in tap water for 5 to 10 min before staining with PAS stain. The PAS stain has a positive reaction with structures containing hexose-sugars and/or sialic acid.

To prepare Schiff's reagent, 0.25% of basic fuchsin was dissolved in distilled water, boiled and then allowed to cool at room temperature. Then 0.25% thionyl chloride ($SOCl_2$) (v/v) was added to the fuchsin solution and the mixture was left under the hood at room temperature overnight. To remove all residual pink color from the mixture, 0.5% of activated charcoal was added and allowed to stir for two or three h or until no pink coloration was observed. To remove charcoal from the solution, a 3M filter paper was used and the filtered solution was stored at 4 ^{o}C in a tightly closed bottle.

The PAS reaction involves several steps: (1) Slides were immersed in 200 ml borohydrate solution [(1% sodium phosphate dibasic (Na_2HPO_4); 0.05% sodium borohydrate ($NaBH_4$)], for 10 min with occasional agitation to release bubbles of hydrogen from surfaces of slides and to reduce aldehyde groups already present in the tissue. Tissue was washed in four changes of tap water. (2) Sections were put in 200 ml of 1% periodic acid solution ($HIO_4.2H_2O$) for 30 to 45 min. This step oxidizes glycol to aldehyde, the

aldehyde are then visualized by reaction with Schiff's reagent (Kiernan, 1990). Stained sections were washed with running tap water. (3) Slides were immersed in 200 ml of Schiff's reagent for 45 min to 1 h and transferred to running tap water for 10 min before staining with Ehrlich's hematoxylin as described above.

Morphometry Cell Counting

Previously accepted procedures (Matikainen et al., 1994; Gaytan et al., 1992; Abney, 1991; Teerds et al., 1989b; Russell et al., 1981; Steinberger, 1973) were used to obtain quantitative evaluation of Sertoli cells (SC), Leydig cells (LC), and myoid cell (MC) numbers. Cross sections were examined by light microscopy using a Zeiss research microscope to assess the quality of tissue preservation prior to quantitative analysis. Random areas of triangular interstitium surrounded by three seminiferous tubule cross-sections were chosen to count LC and MC for each experimental group. This involved counting an average of four interstitial spaces in each of 10 fields per slide from a minimum of four slides per group. At least one slide per animal from each group was examined. LC were recognized by their spherical nucleus with a characteristic distribution of heterochromatin and blue purple staining of cytoplasm, whereas macrophages were identified by their often irregularly shaped nucleus and slightly pink (PAS+) stained cytoplasm (Gaytan et al., 1992; Teerds et al., 1989b).

Sertoli cells were counted when the nucleolus was visible in the section, selecting six seminiferous tubules (ST) for each of the four interstitial spaces chosen for LC and MC in five fields per slide. These criteria for selection result in the counting of cells in 120 tubules per sample. A 40X objective was used to count SC and LC (total magnification 400X); however since MC were too small to count at this power, a 100X objective (oil immersion) was used (total magnification 1000X). When cells were counted, it was necessary to maintain an exact pattern and orientation of the areas where the cells were counted, in order to facilitate an unbiased assessment, and to avoid counting the same cells twice.

Assessment of Seminiferous Tubule and Somatic Cell Diameter

The average diameter of the seminiferous tubules of each animal was determined by measuring the diameter of 25 tubular cross sections by means of image analysis using the MCID-M2 image processing program, Version 2.0, beta 2.1 (Imaging Research Inc.) linked to an IBM computer. Slides were placed under a 10X objective attached to an Olympus microscope, model BX60 with a final magnification of 100X. Only seminiferous tubules presenting a round or slightly oblique profile were quantified (Russell et al., 1981). The image was captured by a digital video camera and displayed in a monitor (MultiSync 6 FG). The outer surface of each seminiferous tubule was traced with a cursor on the television monitor, and from these measurements the diameter of the tubule was calculated by the computer.

A similar protocol was followed to measure the diameter of somatic cells nuclei (SC and LC), using a 40X objective, with a final magnification of 400X. The diameter of MC was not possible to measure, since the nucleus of these cells is very small.

Radioimmune Assay for Serum Hormones

Serum levels of testosterone from irradiated rats control and hormonally treated rat were assayed in duplicate by radioimmunoassay (RIA) using a diagnostic kit (Coat-A-Count) from Diagnostic Products Co. The Coat-A-Count procedure is a solid-phase radioimmunoassay, based on testosterone-specific antibody immobilized to the wall of a polypropylene tube. ^{125}I-labeled testosterone competes for a fixed time with testosterone present in each serum sample. A 50 µl serum sample was placed in labeled polypropylene tubes coated with antibodies to testosterone plus 1.0 ml of ^{125}I testosterone, followed by incubation for 3 h at 37 °C. Additional tubes were processed in duplicate for total hormone count, for control (non- specific binding), and for standard curve of known testosterone concentrations (range between 0 to 16 ng of testosterone/ml). After incubation, each tube was decanted to remove all visible moisture from the tube to enhance the precision of counting. All tubes were counted in a Beckman 5500 *gamma* counter for 1 min.

The calculation of total testosterone was interpolated from alogit-log graph of the standard curve, where the percent bound was plotted on the vertical axis against concentration on the horizontal axis for each of the

calibrator samples. The range of variation of testosterone (stipulated by the manufacturer) is from 2.7 to 10.7 ng/ml. The antiserum used in the kit is highly specific for testosterone (as reported by the manufacturer), with very little crossreactivity to other compounds that might be present in the serum. This procedure can detect as little as 0.04 ng/ml per tube.

Serum FSH and LH were determined using rat FSH and rat LH immunoassay kits purchased from Amersham Life Science. The protocol used was the same for both hormones. Serum samples in duplicate (100µl) were pipetted into labeled polypropylene tubes plus 100 µl of rat FSH or rat LH antiserum (primary antibody) and 100 µl of ^{125}I tracer. Each tube was vortexed thoroughly, sealed with parafilm and incubated overnight at room temperature. Additional tubes in duplicate were processed: (a) for total hormone count (TC), which contained only 100 µl of tracer and considered to be 100% binding for the assay; (b) for control-non specific binding (NSB) containing 200 µl of assay buffer (PBS) plus 100 µl of tracer and no antiserum; (c) for zero standard (Bo) which contained 100 µl of assay buffer (PBS) plus 100 µl of antiserum plus 100 µl of tracer. In addition, known concentration standards ranged from 0.08 ng to 2.5 ng per tube were also processed similarly to the serum sample tubes. After overnight incubation of the tubes, 400 µl of second antibody was added to each tube, except to the TC tube, vortexed vigorously and allowed to incubate for 10 min at room temperature. The antibody bound rFSH and rLH reacted with the second antibody which was bound to magnetizable polymer particles. Separation of the antibody bound fraction in each tube was performed by

centrifugation at 1500 g at 4 ^{o}C. Upon completion of centrifugation, the liquid was aspirated by vacuum from each tube, which was followed by inverting the tubes. Radioactivity of each tube was determined by counting in a Beckman 5500 *gamma* counter for 1 min. A standard curve was constructed by plotting the percent bound of each standard value in the y axis against its concentration value (ng/tube) on the x axis. The concentration of LH and FSH hormone of each serum sample was estimated
from the line of interpolation of the percentage bound and concentration value of each standard plotted in the standard curve.

The rLH assay kit has 100% reactivity to rat LH and <0.016% cross-reactivity to rat FSH. The better sensitivity, as stipulated in the kit is determined by a response at 50% displacement of tracer which is approximately 1.0 ng per tube. The rFSH assay kit has 100% reactivity to rat FSH and <0.17% cross-reactivity to rat LH. The sensitivity is defined as the amount of rFSH needed to reduce zero dose binding by two standard deviations was 0.09 ng/tube.

Protein Synthesis *in vitro*

One sample from each of the four groups, control and treated were incubated simultaneously, under similar conditions of pre-incubation and incubation. The testicular tissue was preincubated to reduce the amount of unlabeled methionine and cysteine. As soon as the testis was decapsulated and weighed, it was immediately minced with iris scissors, suspended in 10 volumes (w/v) of Dulbecco's Modified Eagles' Medium (DMEM; without

methionine and cysteine) containing 3% dialyzed fetal bovine serum, and 1X antibiotic-antimycotic for 1 h at 34 °C, while being gassed with 95% O_2 and 5% CO_2 using a peristaltic pump (Minipuls 3 from Gilson Inc.) to maintain oxygen and pH in the medium. The peristaltic pump controlled the rate of flow of O_2 and CO_2 in each sample, so that percentage of O_2 and percentage of CO_2 were maintained. The flow rate per tube was established by having 10 small bubbles per second in each tube. The controlling of the flow rate, also prohibited the formation of large bubbles which would have displaced the tissue from the incubation medium.

After preincubation, the tissue protein was labeled using the procedure as described below. Each sample was centrifuged at 400 g in the cold for 10 min. The supernatant fluid was discarded and each tissue pellet was resuspended in 10 volumes (w/v) of the same medium used in the preincubation step, plus 100 μCi of Tran^{35}S-label (^{35}S-methionine, ^{35}S-cysteine) reagents purchased from ICN Biomedicals, per ml of medium. Small aliquots (100 μl) of labeled medium were removed from the total medium and from the medium containing each sample for comparison to the uptake of radioactivity per sample at the end of the experiment. Tissue was incubated at 34 °C for 5 h, under similar gassing conditions as in the preincubation step. Preliminary studies in the laboratory, demonstrated that 5 h was the most suitable time for optimal recovery of radioactively labeled proteins from the testis. At the conclusion of the incubation, each sample was centrifuged again at 500 g in the cold for 10 min.

Collection of Labeled Protein and Reduction of Background

Trichloroacetic acid precipitation (TCA) The tissue pellet and supernatant fluid were collected and separated in different centrifuge tubes for further analysis of newly synthesized proteins (Figure 3). To each testis tissue pellet, 2 ml of cold PBS was added and a 100 µl aliquot was set aside and stored at -20 °C. The tissue pellet was then homogenized at 4 °C using a Tissumizer (Tekmar Company). The proteins were dissolved in 2 ml of cold 0.1 N NaOH, and precipitated with 2 ml of cold 10% TCA containing 0.1% DL - methionine. Each tube was vortexed immediately and incubated on ice for 10 min to facilitate precipitation. The precipitate was collected by centrifugation at 1500 g for 10 min at 4 °C. The precipitate was again dissolved in sodium hydroxide and reprecipitated using TCA, vortexed and incubated once more on ice for 10 min. Collection of the newly precipitated product was achieved by centrifugation at 1500 g for 10 min at 4 °C. The NaOH-TCA step was repeated twice for nonspecific background reduction.

To each sample of the medium (interpreted as containing secreted proteins), 2 ml of 1%TCA were added and each sample was throughly vortexed and placed on ice for 10 min. The precipitate from each sample was collected by centrifugation at 4500 g at 4 °C for 10 min. The supernatant fluid was discarded and each protein pellet was dissolved in 2 ml of cold 0.1 N NaOH by homogenization using the Tissumizer. Precipitation steps were similar to the procedure used for those described for proteins from the testis pellet, except

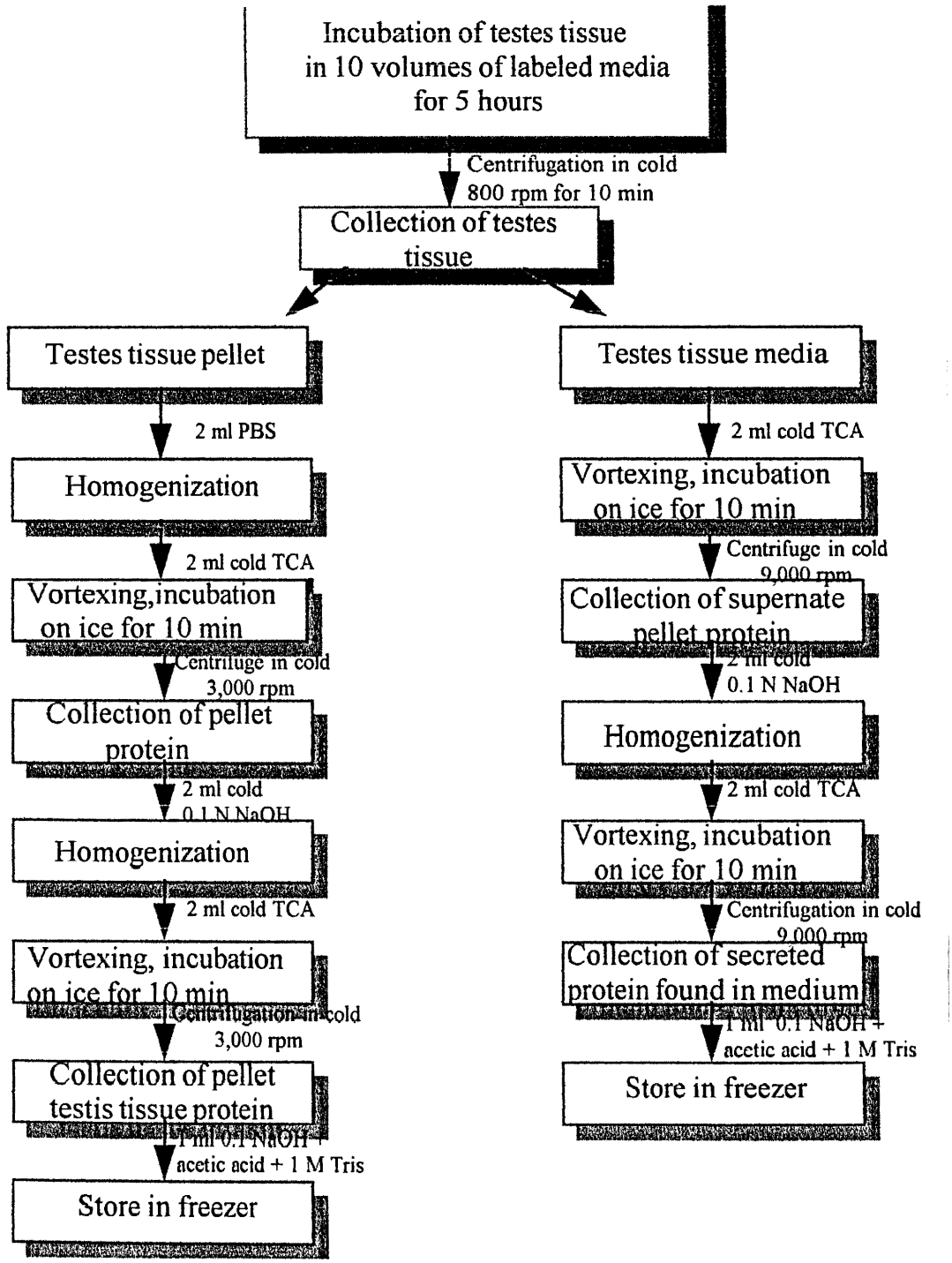


Figure 3. Sequence of steps used to TCA precipitate irradiated rat testes tissue protein

that the centrifugation step was performed at 4500 g. The sodium hydroxide-TCA centrifugation step was also repeated twice for reduction of nonspecific background.

At the completion of the sodium hydroxide-TCA precipitation, proteins from both testis tissue pellet and medium were redissolved in 1.0 ml of 0.1 N NaOH, to which 60 µl of 1 N acetic acid was added to reduce the pH toward neutral and 30 µl of 1 M Tris, pH 7.0-7.6, for the maintenance of pH. Each sample was stored at -20 ^{o}C.

Protein Assay

To determine the specific activity of isotopes incorporated in the newly synthesized protein per µg of total protein, and to establish appropriate sample sizes for gel electrophoresis, protein concentration following TCA precipitation from the testis tissue pellet and the incubation medium was assayed according to Lowry et al., (1951). Bovine serum albumin (BSA) was used as a standard at 1 mg/ml water (0.65 $A_{280\,nm}$/mg protein). The standard curve was generated using 5 µg (5 µl), 10 µg (10 µl), 20 µg (20 µl), 40 µg (40 µl), 60 µg (60 µl), 100 µg (100 µl), 150 µg (150 µl), and 180 µg (180 µl) of protein added to 5 ml tubes in duplicate. Each tube was brought to 200 µl with 0.1 N NaOH. Two additional tubes with only 200 µl of 0.1 N NaOH were prepared and used as a blank sample. Samples of 20 µl from the tissue pellet and an aliquot of medium in duplicate were brought to 200 µl with 0.1 N NaOH. To each tube was added 1 ml of reagent D (2% Na_2CO_3 in 0.1 N NaOH; 1% NaK tartrate; 1% Cu SO_4).

After vortexing each sample was allowed to stand for 10 min and then 0.1 ml of 1 N phenol reagent was added. Each sample was vortexed thoroughly and allowed to stand for at least 30 additional min before measuring the color development with the UV-160 spectophotometer at $A_{660\ nm}$. Concentrations were calculated from interpolation on the standard curve.

Radioactive Protein Determination

The TCA precipitable radioactivity of 20 µl aliquots was determined in each testis sample with a Beckman LS 9000 scintillation counter. A calculated dilution factor was used to determine the total radioactivity incorporated into each sample. The specific activity (counts per minute/µg of protein) of the total cpm from each testis pellet and precipitate from medium was determined by dividing cpm/20µl of each sample with the µg of protein/20µl of each respective sample.

Separation of *in vitro* Synthesized Protein by size using Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Radiolabeled proteins synthesized by testicular cells from various treatment groups were separated by molecular weight using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed using slab gels containing 10% polyacrylamide in the separating gel and 5% polyacrylamide in the stacking gel (Mills and Ilan, 1985).

Preparation of gels

(a) Slab gel: 1X of lower chamber Tris (0.4 M Tris-HCl, 0.1% sodium dodecyl sulfate (SDS)), acrylamide solution (10% acrylamide, 1.5% diallyltartardiamide (DATD) crosslinker, 0.1% of linear polyacrylamide), 0.06% of ammonium persulfate solution, 0.025% of tetraethylmethylenediamine (TEMED), and 48.5% of deionized water were added to make 40 ml as a final volume. Water was mixed with lower chamber Tris, acrylamide solution and with ammonium persulfate, except TEMED, which was added last. The mixture was mixed thoroughly, avoiding the formation of air bubbles. A gel casting system which consisted of two glass plates (18 cm X 20 cm) placed together face to face, but separated by a 1.5 mm spacer and secured by binder clips was assembled in advance. Melted agarose (1%) was used to seal the edges of the glass plates in order to avoid leaks. The slab gel solution (40 ml) was gently mixed and quickly poured into the slab gel cast. The glass was tapped after filling to remove any trapped air bubbles. Water was overlayed very slowly on top of the gel mixture to form a flat interface for the stacking gel. Polymerization occurred in 20 to 30 min, and the overlayed water was discarded.

(b) Stacking gel: A 20 ml solution of 61.5% deionized water, 1X upper chamber Tris (0.13 M Tris-HCl, 0.1% SDS), acrylamide solution (5% acrylamide, 0.75% diallyltartardiamide, 0.05% of linear polyacrylamide), 0.04% of ammonium persulfate, and 0.1% of TEMED was prepared. As in preparation for the slab gel, all of the components were added except TEMED. This mixture

was used to rinse the top of the slab gel, then it was removed. After addition of TEMED, the mixture was gently mixed and poured on top of the slab gel. A teflon comb was carefully placed between the top and inside of the two glass plates to form wells in the stacking gel as polymerization occurred. The comb was removed after polymerization and each well was rinsed and filled with a well buffer prior to loading the protein samples.

Sample Preparation

Sample buffer- tracking dye (5% glycerol, 2.5% 2-mercaptoethanol, 1.5 M urea, 5% SDS, 0.2% bromphenol blue, 0.5% phenol red) was added to 20 μg of [^{35}S] labeled protein and heated at 90 ^{o}C for about 2 min, cooled and heated again for about 1 min. Once each sample was cooled, it was loaded in each well underneath the well buffer present in the well. Each run included gels from both control and treated rats. A mixture of molecular weight standards prepared in the laboratory was also used in each electrophoretic run, and the components of the mixture were as follows (kDa): cytochrome C (12.3k), Rnase B (14.7k), glucose-6-phosphate dehydrogenase (36.5k), ovalbumin (43.5k), albumin (67k), rat transferrin (76k), Phosphorylase B (94k), and B-galactosidase (116k).

Electrophoresis

Electrophoresis was conducted using 1X Tris-glycine buffer (0.3% Tris base ultrapure, 1.45% glycine) pH 8.8, and 0.1% SDS loaded in the upper and

lower chambers of the electrophoretic apparatus. Samples were electrophoresed into the stacking gel at 80 volts. The migration of the tracking dye, was monitored and once the tracking dye has entered the slab gel, the voltage was increased to 110 volts. Each electrophoresis run was performed at room temperature for 6 h. Electrophoresis was stopped when the tracking dye was within one centimeter of the bottom of the gel.

Fixing and Staining the Gel

To fix the separated proteins in location, after electrophoresis, gels were removed from glass plates and placed in 200 ml of fixative solution (20% methanol, 7% glacial acetic acid). The fixation step is important for minimizing the amount of swelling and distortion of the gel matrix during fixing and staining. The first change of fixative was discarded after 1 h and the gel was fixed overnight in the second change of fixative (400 ml). After fixative removal, gels were placed in 200 ml of G-250 coomassie blue staining solution (0.04% coomassie blue G-250, 10% methanol, 2.8% perchloric acid) for 45 min and rinsed several times with tap water. To eliminate staining background, the gel was placed in several changes of fixative until no specific background was seen in the gel. The last change of fixative was discarded and 200 ml of drying solution (20% methanol, 2% glycerol) was added to the gel twice for 30 min each to remove acetic acid from the gel. This step was also designed to maintain the stability of the matrix (methanol regulate the hydration of the gel) of the gel.

Drying the gel

Gel was taken out from the drying solution and placed on 3M paper, which was cut to a size similar to that of the gel and immediately covered with Saran wrap. The drying paper containing the gel was placed on a flat surface of a drying apparatus connected to a vacuum pump with side arm flask for a vapor trap to collect the water removed from the gel which was heated to 75 ^{o}C for 2 h.

Autoradiography

Dried gels were taped into X-ray cassette holders (21 cm x 26 cm) and then exposed to X-omat X-AR 5 film (Kodak). Initial exposures were for about 48 h and then an estimate of additional time required was made, usually about 5-6 days.

After 6 days of exposure, the film was developed in 300 ml of Kodak developer (HC-110) in 1:7 (stock solution:water) in the dark for 7 min. The developing process was terminated in a stop bath for 30 seconds, followed by 3 min in rapid fix solution from Kodak. The last step was performed with normal light. Film was rinsed several times with tap water and allowed to dry prior to analysis in the image analyzer.

Analysis of Autoradiograms by an Image Analyzer

Image Analysis was performed using the MCID-M2 Operation system, version 2.0, Beta 2.1 (Imaging Research Inc). Autoradiograms were placed on

a stabilized transillumination light pad (Northern light box from Imaging Research, Inc.). The image was captured by a digital video camera (MultiSync 6 FG), which displays the image on a monitor using the MCID-M2 operation system, Version 2.0, Beta 2.1 loaded in an IBM computer (Max Sys, CSS Laboratories, Inc). The IBM computer was also attached to a host monitor which displays programs and computer functions (MultySync 5 FG).

Relative optical density (ROD) image processing is a mathematical operation which changes the appearance of a digital image by transforming the gray level values of the pixel which comprise the image. The image was processed and analyzed using one-dimensional, gel analysis software (Imaging Research, Inc.). The background (baseline correction) was set manually for each X ray film. The average of the ROD values obtained from six random measurement of each corner of the X ray film was used as a background value per each film. The background value (ROD) was subtracted from each ROD value obtained from each band present in the respective autoradiogram. The determination of the background allowed the evaluation of the intensity of each band, by means of obtaining the relative ROD value of each band after background subtraction.

A complete set of autoradiograms (6) from each separate experiment was analyzed using similar parameters, established for the capture and processing of the image.

Values of respective bands processed by the image analyzer to compute both their relative optical density and the percentage of total density from the

respective lane in which the band was identified. Only the more prominent bands of newly synthesized proteins were analyzed using image analysis.

Statistical Analysis

Statistical analyses were performed on SPSS (Statistical package for the social sciences) program (Version 4.0) available on the mainframe computer at Texas Woman's University. Results were tested for statistical significance using one-way analysis of variance (ANOVA). Tukey's test was used for post-hoc evaluation of means among groups. Differences were considered significant if $p<0.05$.

CHAPTER III

RESULTS

This study was designed to demonstrate the effect of testosterone on the morphology of somatic cells of the rat testis following testosterone withdrawal or replacement. The morphometric evaluation, in turn, provided an excellent framework in which to study the protein profile of the testis under experimental conditions. Evaluating these two parameters, morphometry and protein profile, may have further implications in the understanding of spermatogenesis in mammals, which is under testosterone control. Many types of cell populations inside and outside of the seminiferous tubules are believed to play an important role in spermatogenesis, but by focusing only on the somatic cells, it can be demonstrated that their function is necessary for normal development of spermatogenesis.

Many studies of the effect of testosterone in the testis exist in the literature (Sharpe et al.,1992; Awoniyi et al.,1989; Santulli et al.,1990), but most of them have been performed in isolated cell populations grown in culture (Sharpe and Cooper,1982; Skinner and Fritz,1985). The present study which was performed *in vivo*, uses two models. The first involved the elimination of germ cells by X-irradiation of males *in utero*, which also removed the proteins and other products produced by these cells. The second model

involved the withdrawal of testosterone in the testes, which was accomplished by the administration of Leuprolide, a GnRH agonist. The effect of Leuprolide is to block the release of luteinizing hormone (LH) from the pituitary which, in turn, impairs the production of testosterone by Leydig cells. This model allowed analysis of changes in both somatic cell morphology and protein profiles in the presence or absence of testosterone. Additional parameters that provided important information for the interpretation of these results included body and testis weight and measurement of hormone levels in serum such as luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone (T). Therefore, the *in vivo* approach used in this study in which germ cells (via irradiation) and endogenous gonadotropin and testosterone (via a GnRH agonist, Leuprolide) were eliminated provided an opportunity to investigate the effect of testosterone on somatic cells. The morphological and biochemical responses of these cells to the administration of Leuprolide alone or testosterone alone, or in combination of both, were tested.

Body Weights

No significant differences (Table 1) were observed in the final body weights (grams) of irradiated rats treated with various treatments when compared to that of the irradiated control group. No reduction in body weight was observed in any group during the treatment, which suggests that animals were not significantly affected by any treatment and were considered healthy for the study.

Table 1

Initial and final body weights of irradiated male rats (Fisher 344) treated with Leuprolide (Leupr.), testosterone propionate (T.P.), or with both Leuprolide and testosterone propionate

Treatment	n	Body weight (grams) Initial Mean ± SE	Final Mean ± SE	Change
Control	(6)	237 ± 9.0	277 ± 7.3	+16.8 %
Leupr.	(6)	227 ± 10.4	252 ± 6.9	+11.0 %
T.P.	(6)	228 ± 10.9	245 ±10.3	+ 7.4 %
Leupr.+ T.P.	(6)	237 ± 7.9	259 ± 5.2	+ 9.3 %

n = number of animals per group.

Rats were treated with a depot injection of Leuprolide (LHRH agonist), androgen (testosterone propionate, 10 mg/kg body weight, every 3rd day) or both Leuprolide and testosterone propionate. The treatment period was for six weeks and controls were treated with equal volumes of vehicle for GnRH and sesame seed oil to match the solvent and volume for testosterone propionate.

Testis Weight

Germ cells make up 80% of the total testis weight in normal rat testis. Since each male rat was *gamma*- irradiated *in utero* with 180 rads, which eliminated all germ cells present in the testes, it was expected that these rats would have small testes when compared with their non- irradiated counterparts. Testicular weight from one testis of the irradiated rats was used since a small portion of the other testis was used for morphological analysis. The weight of the irradiated testis (with tunica albuginea) treated with Leuprolide was significantly decreased ($p<0.05$; 53%), when compared to those of irradiated control animals (Table 2). The testis weight of testosterone alone treated animals (T.P.) and testosterone in combination with Leuprolide treatment (Leupr. + T.P.) was reduced 32% and 46% respectively. When the tunica albuginea was removed from the testes, decreases of 52%, 31%, and 44% were observed in the Leuprolide, testosterone and in the combination treated group, respectively when compared to the control group. Testosterone treatment with or without Leuprolide did not maintain testicular weight; but greater testis weight was observed in the animals treated with testosterone plus Leuprolide when compared to those treated with Leuprolide alone.

Testicular Morphology

To assess changes in tissue of treated animals, cell populations were evaluated histologically. Testicular cross sections were examined by light microscopy to judge the quality of tissue preservation, identify somatic cells

Table 2

Testicular weight of irradiated rats (Fisher 344) treated with Leuprolide (Leupr.), testosterone propionate (T.P.), or with both Leuprolide and testosterone propionate

Treatment	n	Testis			
		With tunica albuginea (g)	% of control	Without tunica albuginea (g)	% of control
Control	(6)	0.28 ± 0.09	100	0.23 ± 0.04	100
Leupr.	(6)	0.13 ± 0.04*	47	0.11 ± 0.03*	48
T.P.	(6)	0.19 ± 0.07*	68	0.16 ± 0.05*	70
Leupr.+ T.P.	(6)	0.15 ± 0.02*	54	0.13 ± 0.04*	56

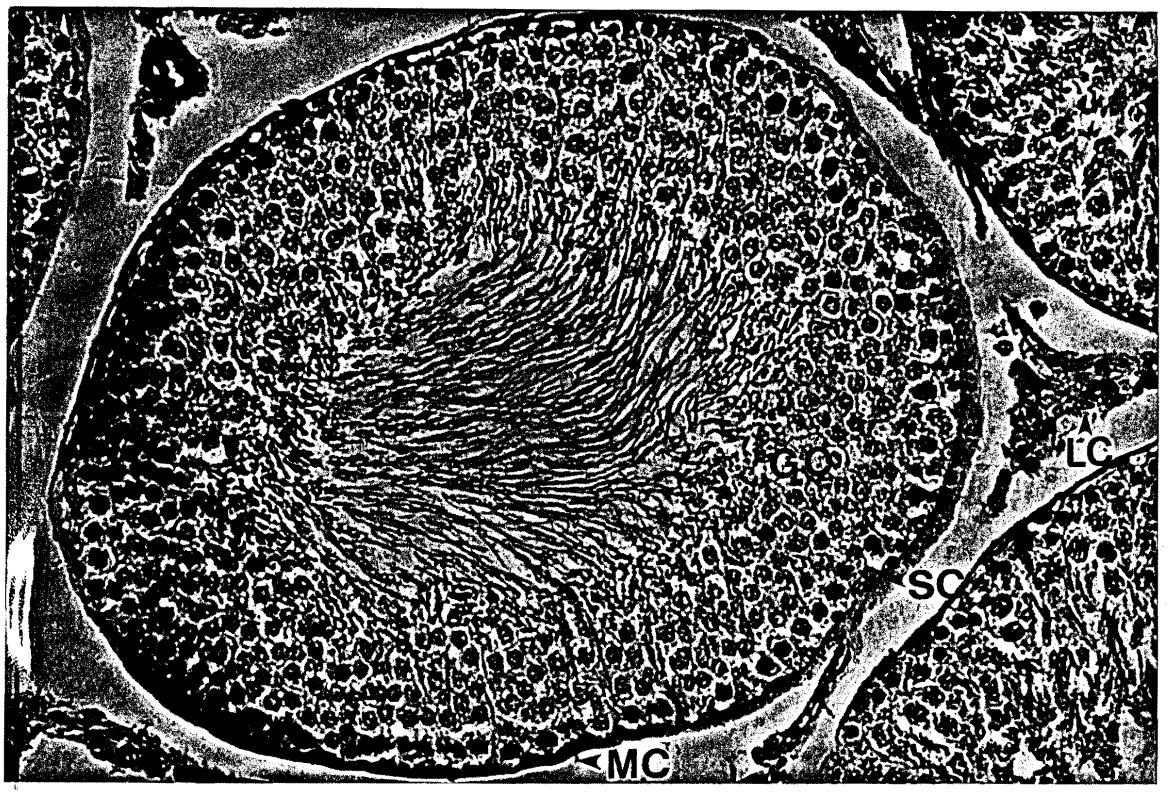
* Marks significant differences ($p<0.05$, Tukey test) when compared to controls.
n= Number of animals per group

Each testis was weighed with and without the tunica albuginea as soon as they were detached from each rat. Only the weight of one testis/rat was used for comparison since a small piece of the second testis was immediately taken with the tunica albuginea intact for histological analysis.

prior to quantitative analysis and verify the absence of germ cells in the seminiferous tubules as a result of irradiation, in order to show that germ cells were not contributing to the protein profile.

To show the difference between normal and germ cell free rats we evaluated the histology of both normal testes and testes of germ cell free animals. In the normal rat testis (Figure 4 A and Figure 4 B), germ cells (GC) are well defined in the adluminal space inside the seminiferous tubules, and represent about 80% of the cell mass. The somatic cells in the normal testes are Sertoli cells (SC), myoid cells (MC), Leydig cells (LC) and macrophages (M). The nuclei of the SC, which are located alongside the basement membrane of the seminiferous tubules, are easily identified by their shape and large nucleoli. Myoid cells are distributed outside and around the seminiferous tubules and appear to consist of only one layer of cells. Leydig cells and macrophages are located in the interstitial space (IS). Leydig cells are very distinct, variable in shape (round or oval) with a characteristic punctate pattern of irregularly distributed heterochromatin. Macrophages are very conspicuous in the interstitial space having visible pink cytoplasm as a result of a positive reaction with the periodic acid Schiff stain (PAS).

In irradiated rats which are devoid of GC, the somatic cells are more prominent and SC become the dominant cell type (Figure 5 A and Figure 5 B). One important feature in the irradiated control group was the IS in which the LC appeared to have increased in size (hypertrophy).



4 A. Light micrograph of a cross section of normal rat testis seminiferous at 825X magnification

normal rat testis is used to compare its morphology to those of irradiated rat Sections of 7 μm thickness were cut and stained with periodic acid Schiff and counterstained with Ehrlich's hematoxylin. Germ cells (GC) and cells (SC) are inside the seminiferous tubules, myoid cells (MC) the lamina propria, and Leydig cells (LC) are in the interstitial space.

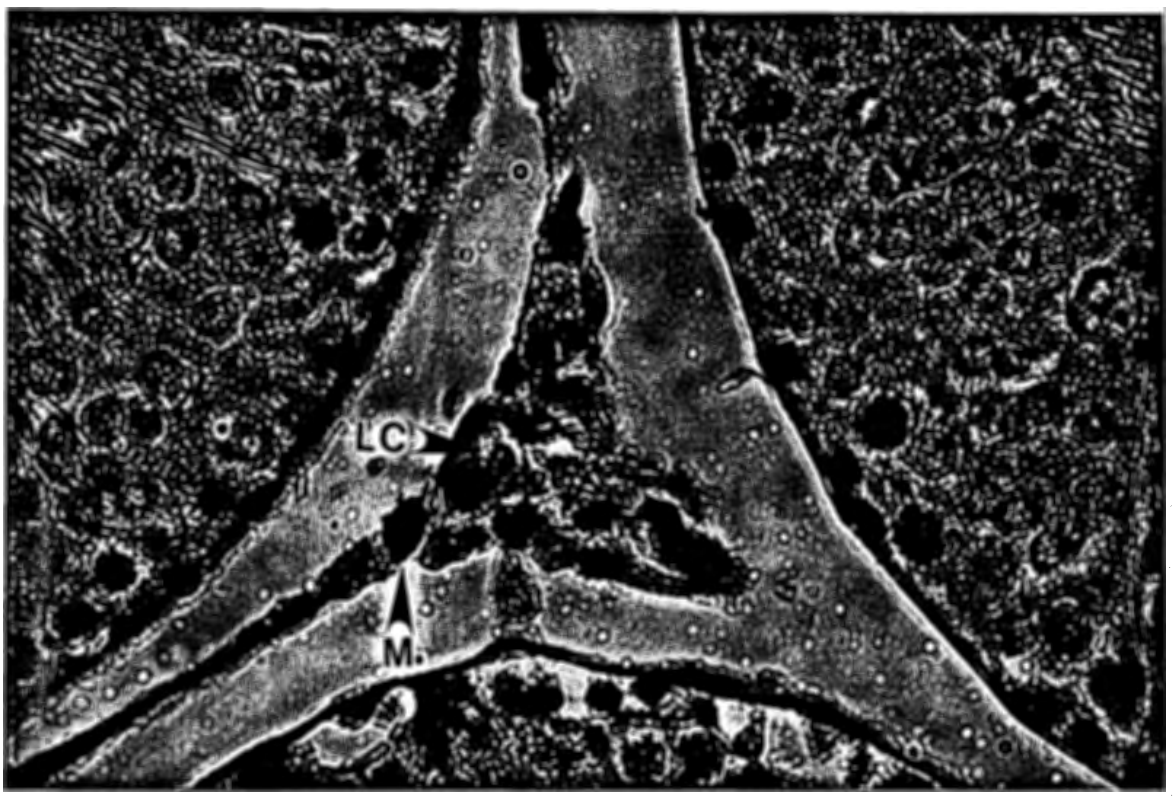


Figure 4 B. Light micrograph of normal rat testis interstitial space at 2000X magnification

Sections of 7 μm thickness were stained with PAS and counterstained with Ehrlich's hematoxylin. Leydig cells (LC) are identified by a punctate distribution of heterochromatin. Macrophages (M) are identified by a pink cytoplasm as a result of their positive reaction with PAS stain.

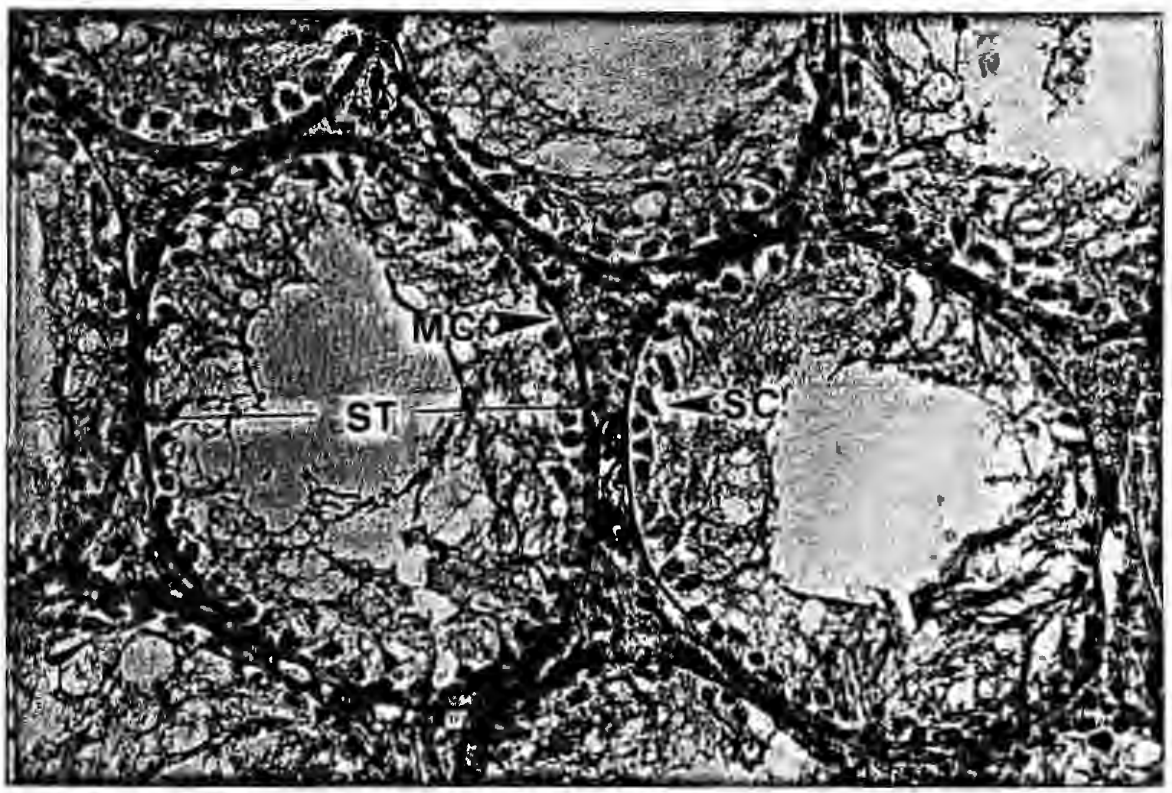


Figure 5 A. Light micrograph of a cross section of Irradiated rat testis at 825X magnification

Sections of 7 µm thickness were stained with PAS and counterstained with Ehrlich's hematoxylin. A reduction of the seminiferous tubule (ST) diameter is evident when compared to those of normal rat testis. Germ cells are absent and the predominant cell is the Sertoli cell (SC). Hence, this rat testis model is known as Sertoli cell enriched testis (SCE). Previous studies have demonstrated that irradiation alone does not alter SC and myoid cell (MC) nuclear morphology.

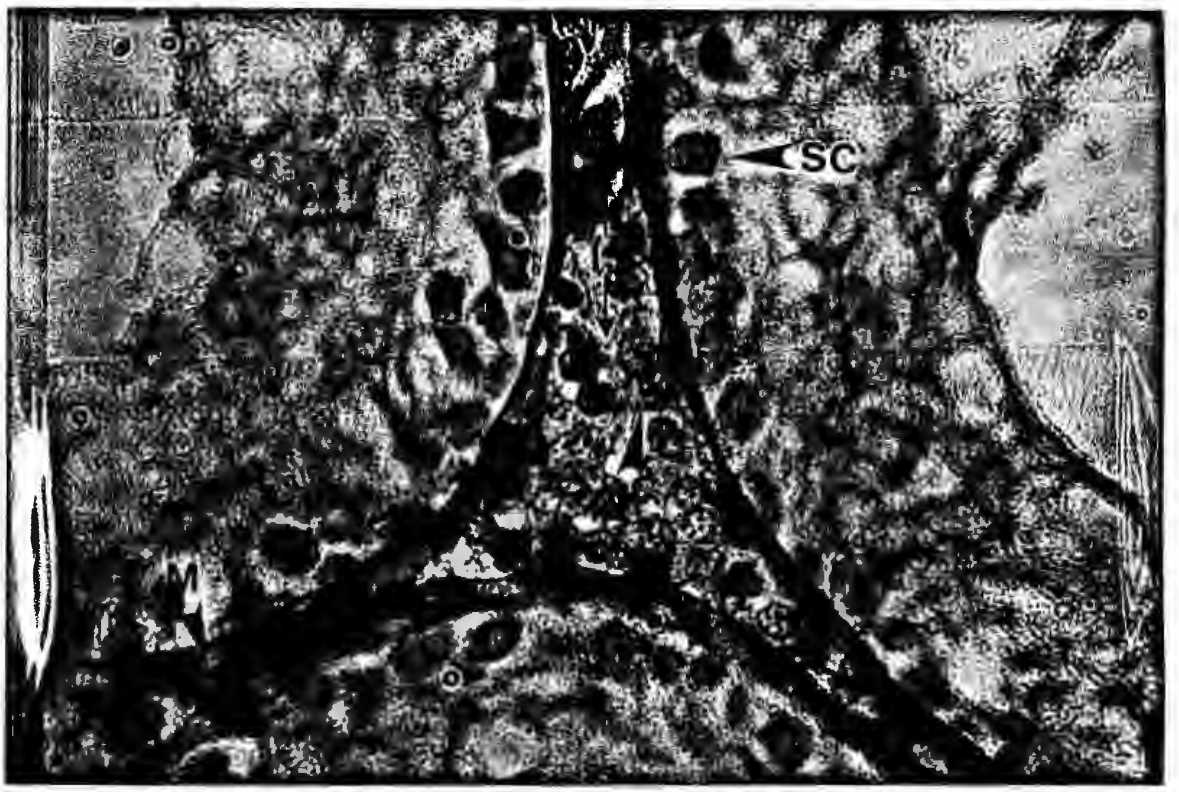


Figure 5 B. Photomicrograph of interstitial tissue from irradiated rat testis at 2000X magnification

Sections of 7 μm thickness were stained with PAS and counterstained with Ehrlich's hematoxylin. Sertoli cells (SC) nuclei are alongside the basal membrane of the seminiferous epithelium with similar morphology that those observed in the normal rat. Leydig cells (LC) are characterized by spherical nuclei and prominent distribution of chromatin. Macrophages (M) are identified by their irregularly shaped nucleus and pink cytoplasm (PAS+). Interstitial space (IS) and LC are greater portion of total testicular tissue when compared to the normal rat.

Similar observations were demonstrated by Rich et al. (1979). Schoen (1964) has speculated that LC hypertrophy is due to the compensatory mechanism of the cell, since there are no GC in these testes. The alteration of testicular morphology with the Leuprolide treated group (Figure 6 A) is evident mainly in the LC and in the IS itself. Cells that resemble LC with their characteristic patches of heterochromatin but with a fusiform morphology, are visible in the IS. Likewise, the IS is reduced in size and is filled with intensively stained interstitial fluid which is better visualized at higher magnification (Figure 6 B). Numerous fusiform, elongated cells, without patches of heterochromatin are observed in the IS but the normal identifiable features of LC could not be identified by light microscopy. Many cells at different stages of degeneration were also observed in the interstitial space. No morphological changes of SC nuclei were observed, when compared to the control group, but many of them were detached from the basement membrane but were still present in the lumen of the seminiferous tubule. MC were arranged side by side to the basal lamina of the seminiferous tubules with a fusiform and elongated nucleus. No light microscopic changes in their morphology were observed when compared to the control group.

In testosterone treated rats (Figure 7 A and Figure 7 B) some LC maintained their normal morphological characteristics. Most somatic cells remained in their normal locations. The nuclear morphology of SC in this treated group did not differ from those of the control group, but the nucleoli appear to be more dense

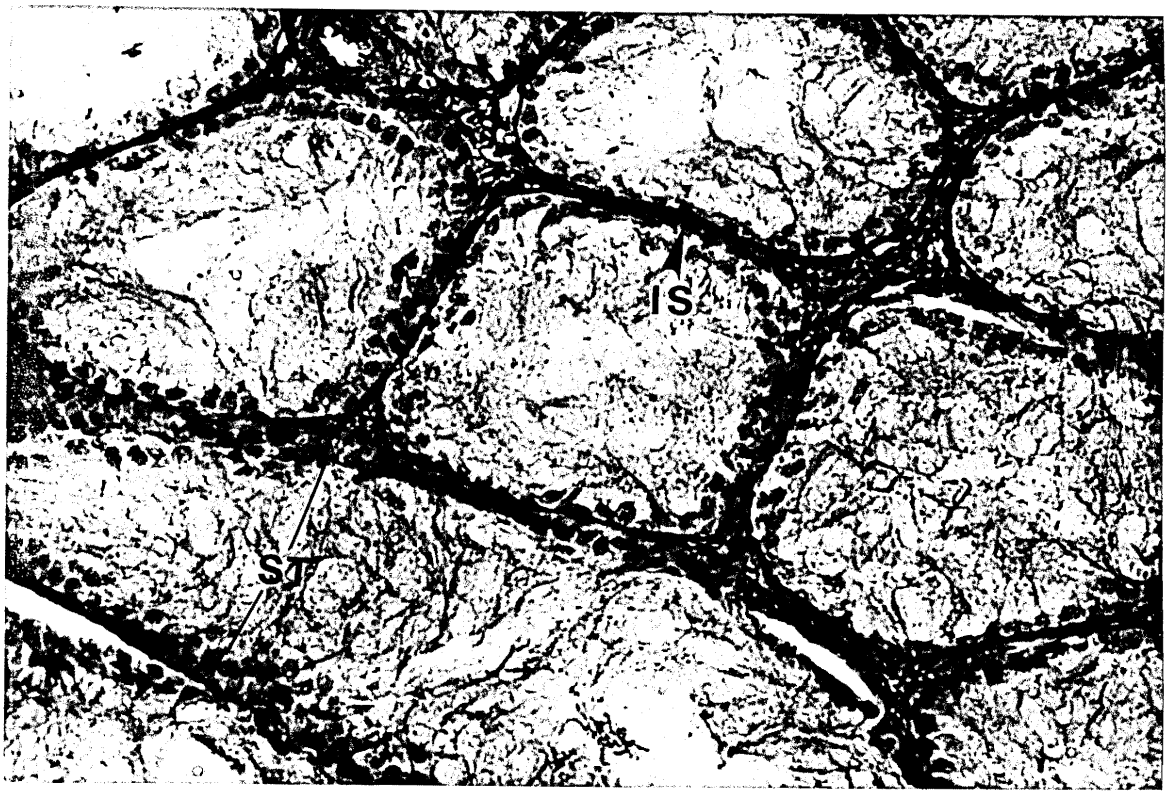


Figure 6 A. Photomicrograph at 825X magnification from irradiated rat testis treated with a GnRH agonist (Leuprolide) for 6 weeks

Animals were treated with 100 µg/kg body weight per day of Leuprolide in microspheres as a depot injection subcutaneously. Sections of 7 µm were stained with PAS and counterstained with Ehrlich's hematoxylin. A reduction of the seminiferous tubule diameter (ST) makes the tissue appear as a sheet-like structure. Interstitial space (IS) is reduced and filled with numerous cells.

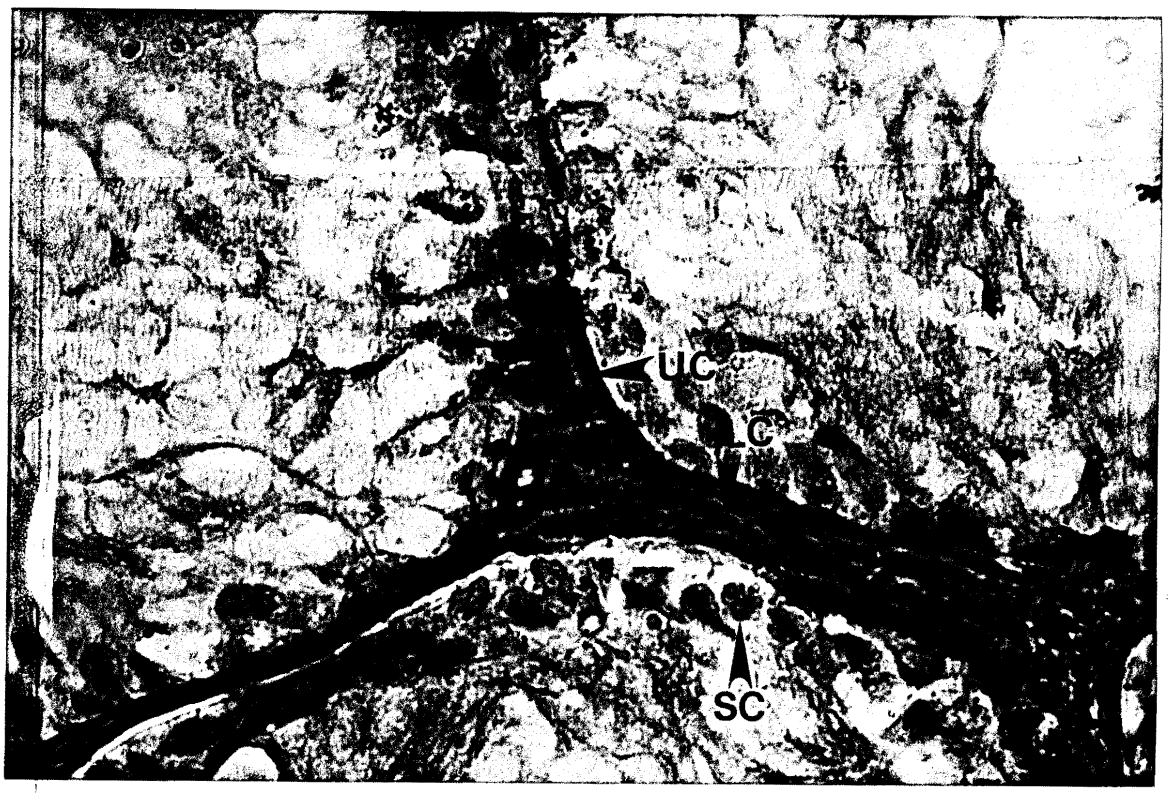


Figure 6 B. Photomicrograph of interstitial space (2000X magnification) from irradiated rat testis treated with a GnRH agonist (Leuprolide) for 6 weeks

Sections of 7 μm thickness were stained with PAS and counterstained with Ehrlich's hematoxylin. LC nuclear morphology has changed but still have a fusiform and elongated shape, however the punctate heterochromatin pattern is still viewable. Unidentifiable interstitial cells (UC) are seen in the interstitial space. Some nuclei of Sertoli cells (SC) are detached from the basal membrane.

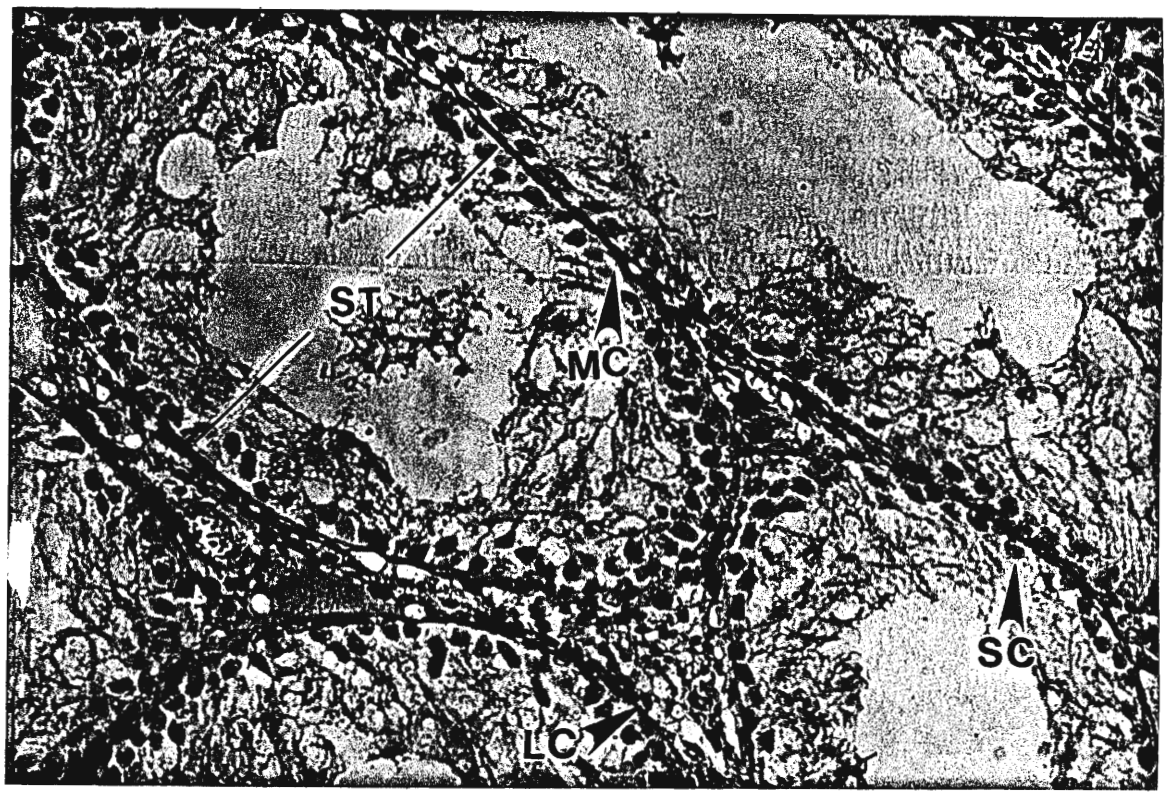


Figure 7 A. Photomicrograph of cross section of irradiated rat testis treated with testosterone propionate at 825X magnification

Sections of 7 μm thickness were cut and stained with PAS and counterstained with Ehrlich's hematoxylin. Seminiferous tubule diameter (ST) was not maintained with this treatment. Most of the Sertoli cells (SC), myoid cells (MC) and Leydig cells (LC) remained in their normal locations.

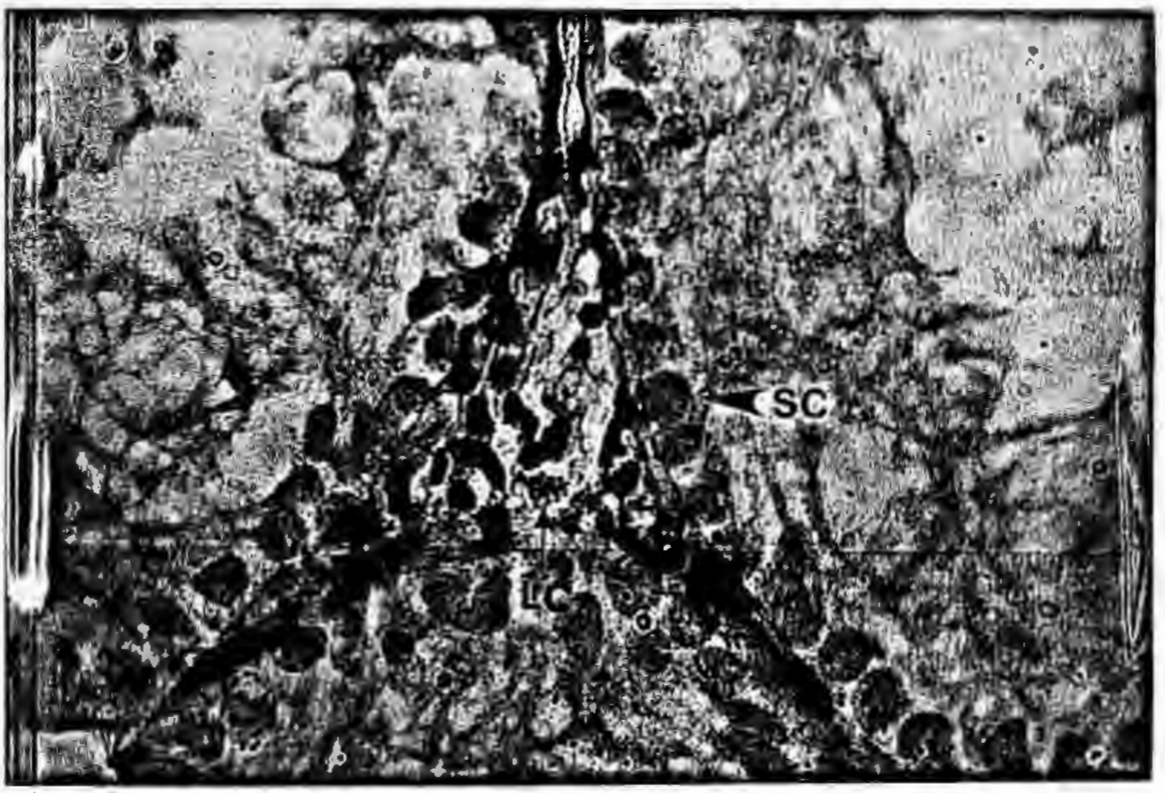


Figure 7 B. Photomicrograph of interstitial tissue from irradiated rat testis treated with testosterone propionate viewed at 2000X magnification

Sections of 7 μm were stained with PAS and counterstained with Ehrlich's hematoxylin. Some Leydig cells had normal morphological pattern of chromatin (arrow) with testosterone propionate treatment. Sertoli cell (SC) nuclei unaffected.

when compared to the Leuprolide treated group. Testosterone alone induced alteration in the normal morphology of LC.

In animals treated with a combination of testosterone propionate and Leuprolide (Figure 8 A) the morphological alteration of LC was still evident, as well as the presence of numerous unidentified cells in the IS. This is more readily visualized at higher magnification (Figure 8 B). Testosterone replacement did not overcome the effect of Leuprolide in the testicular tissue, since the morphology of LC still resembles the morphology observed in the Leuprolide alone treated group. No changes in the nuclei and nucleoli of SC or in the morphology of MC nuclei were observed, when compared to the control group.

In summary, the absence or presence of androgen appeared to make little difference in the nuclear morphology of SC and MC. The morphology of LC, on the other hand, was drastically altered with these same treatments.

Diameter of Seminiferous Tubules and Somatic Cells

The diameters of the seminiferous tubules (ST) and somatic cells obtained using an image analyzer revealed differences between controls and Leuprolide treated rats (Table 3 and Figure 9). Some ST presented a distorted shape which made it difficult to take the measurement. Consequently, only circular profiles were considered in each group, as suggested by Teerds et al. (1989a) and Misro et al. (1992). This criterion maintained the random measurement of tubules. The possibility that some tissue shrinkage occurred

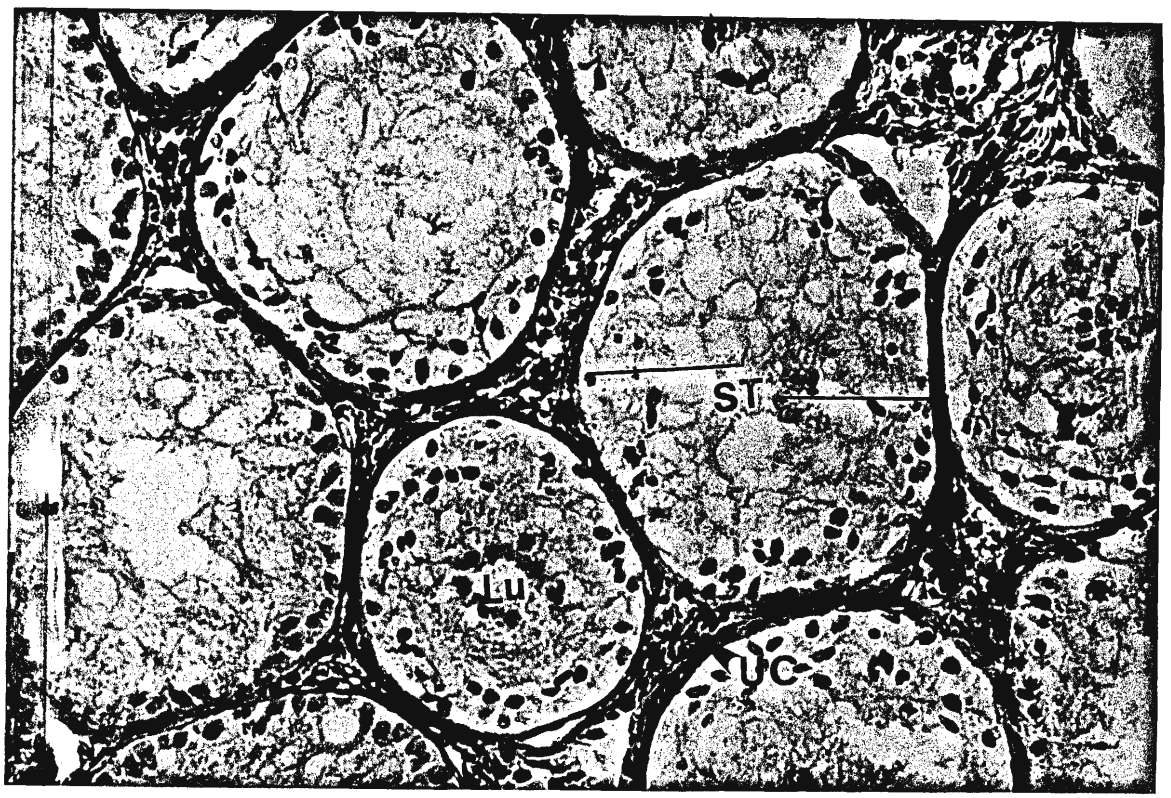


Figure 8 A. Photomicrograph of cross section (825X magnification) of irradiated rat testis treated with Leuprolide plus testosterone propionate

Each animal received 100 µg/kg body weight per day of Leuprolide in microspheres as a depot injection subcutaneously for 6 weeks. Testosterone propionate was dissolved in sesame seed oil (SSO) and administered at 10 mg/kg of body weight at 3 day intervals for 6 weeks. Sections of 7 µm were stained with PAS and counterstained with Ehrlich's hematoxylin. A reduction of the seminiferous tubules (ST) was still evident. The interstitial space (IS) was still filled with unidentified cells (UC).

8 B. Photomicrograph of interstitial space from irradiated rat testis with Leuprolide plus testosterone propionate viewed at 2000X

of 7 μm thickness were stained with PAS and counterstained with hematoxylin. Leydig cells (LC) morphology still show the effects of in the presence of testosterone. Some Sertoli cells (SC) are in the lumen (Lu) as a result of their apparent detachment from the membrane.

Table 3

Diameters of seminiferous tubules and somatic cell nuclei of irradiated rat testes under different treatments

Treatment	Seminiferous Tubules (μm)	Sertoli cells Nucleus (μm)	Sertoli cells Nucleolus (μm)	Leydig cells Nucleus (μm)
Control	163.0 ± 1.3	6.9 ± 0.16	1.6 ± 0.01	4.9 ± 0.06
Leupr.	115.0 ± 1.1*	6.4 ± 0.15	2.1 ± 0.08*	2.9 ± 0.07*
T.P.	138.0 ± 0.8*	6.8 ± 0.15	1.7 ± 0.02	3.6 ± 0.07
Leupr.+T.P.	128.0 ± 2.2*	6.8 ± 0.23	1.8 ± 0.1	3.1 ± 0.07*

(*) Marks significant differences ($p<0.05$, Tukey test) when compared to controls.

The diameters of seminiferous tubules, Sertoli cells and Leydig cells were measured using an image analyzer system. Random areas of the triangular interstitium surrounded by three seminiferous tubule cross- section was chosen for each experimental group. Averages were calculated for each treatment group by compiling the values obtained from all testes of the same group (n=6).

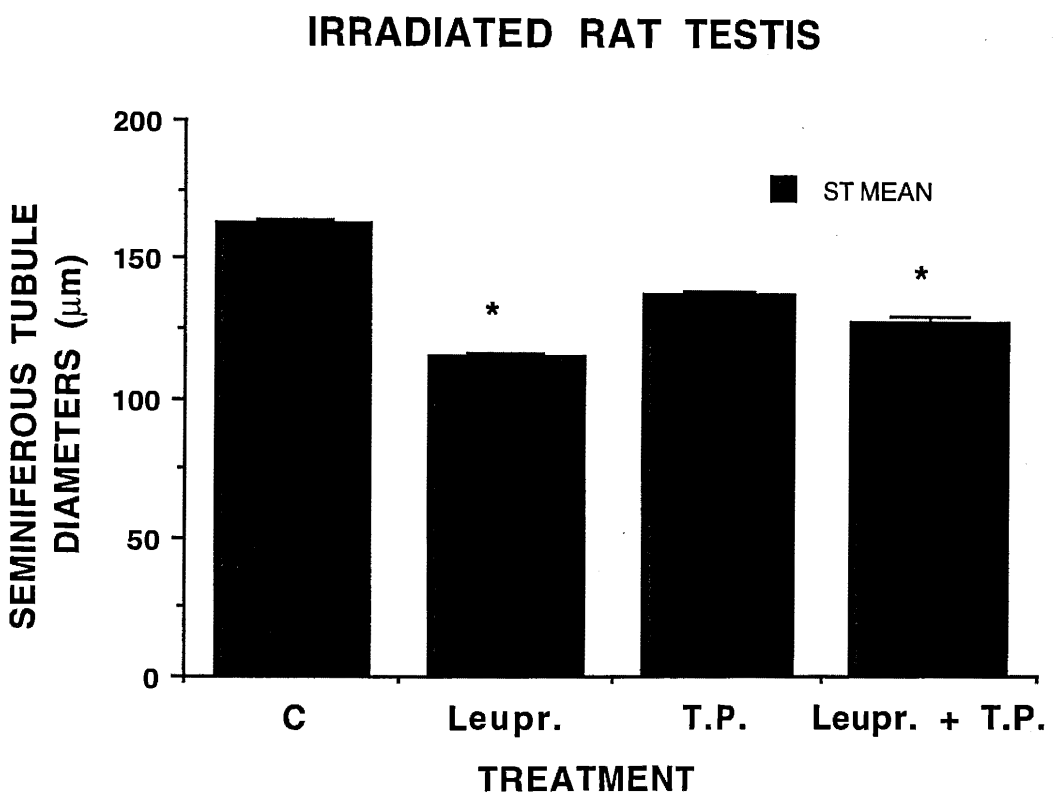


Figure 9. Seminiferous tubule (ST) diameters (μm) of irradiated testes under different treatments

These data are taken from Table 3.
* Marks significant differences ($p<0.05$, Tukey test) when compared to controls.
Treatments are: control (C); Leuprolide (Leupr.); testosterone propionate (T.P.); and Leuprolide + testosterone propionate (Leupr. + T.P.).

as a result of the fixation can not be discounted. However, all tissues were treated under the same conditions of fixation, embedding and sectioning. We assume, therefore, that the difference of ST diameter were most likely due to the different treatments used in the study.

ST diameters of the Leuprolide treated rats (115 ± 1.1, $p<0.05$) were reduced by 30% when compared to those of the irradiated control group (163 ± 1.3, $p<0.05$). Likewise, a 15% and 22% reduction was observed in the testosterone treated group with or without Leuprolide (138 ± 0.8; 128 ± 2.2, $p<0.05$), respectively, when compared to those of the control group. Since tubular diameter, like LC morphology, was not maintained by the replacement of testosterone, other tubular or paracrine factors subjected to stimulation by hormones different than testosterone or Leuprolide may be involved in maintaining the diameter of both ST and LC and the normal morphology of the latter.

The nuclear diameter of SC did not change in any treated group when compared to the control values and the ranges are between 6.4 μm and 6.9 μm (Table 3 and Figure 10). On the other hand, nucleolar diameter of SC were affected by the Leuprolide treatment with an increase of 31% (2.1 ± 0.08, $p<0.05$), compared to those of the control group (1.6 ± 0.01, $p<0.05$) and to other treatment groups.

Nuclear morphology of LC was drastically affected by Leuprolide treatment, and the administration of testosterone did not maintain their morphology (Table 3 and Figure 11). This observation was corroborated by

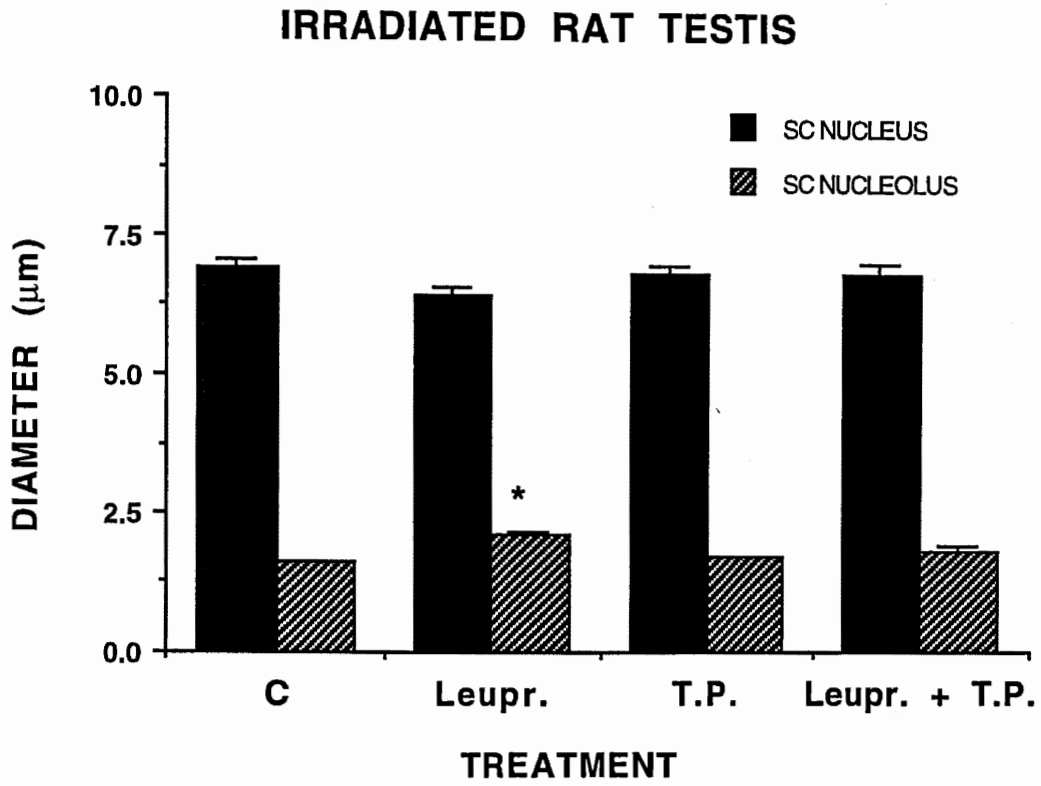


Figure 10. Morphometry of Sertoli cell (SC) nuclei and nucleoli

The data are from Table 3.
* Marks significant differences ($p<0.05$, Tukey test) when compared to controls.
Treatments are: control (C); Leuprolide (Leupr.); testosterone propionate (T.P.); and Leuprolide + testosterone propionate (Leupr. + T.P.)

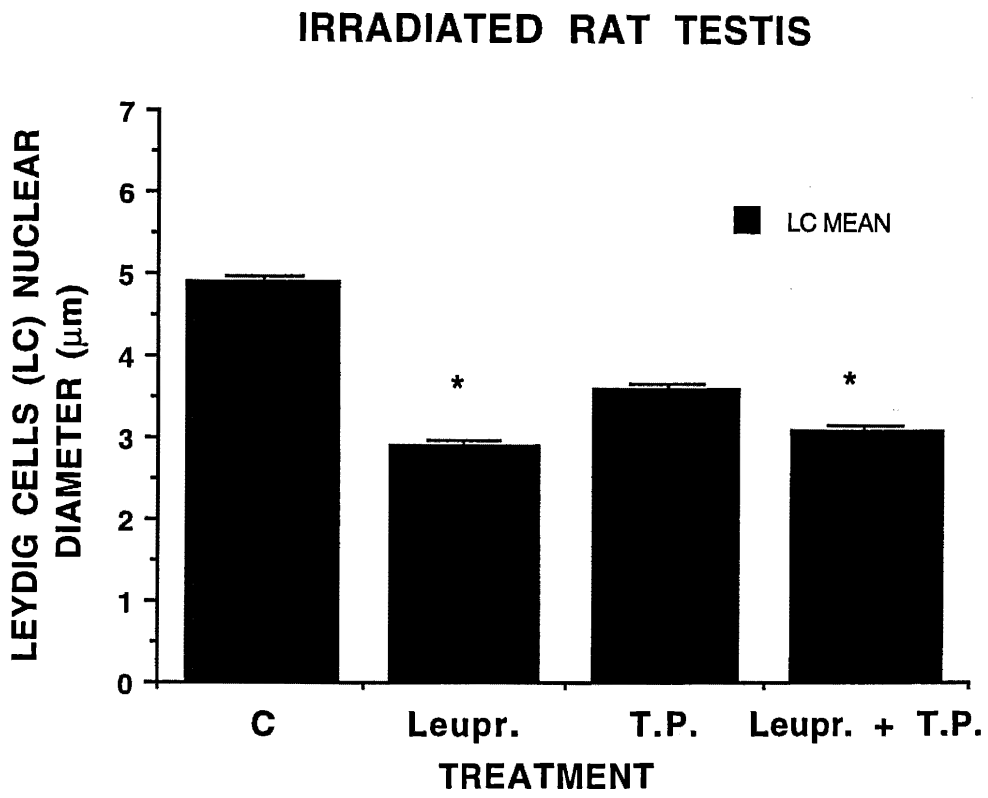


Figure 11. Leydig cell nuclear diameters (µm) of irradiated rat testes

These data are from Table 3.
* Marks significant differences ($p<0.05$, Tukey test) when compared to controls.
Treatments are: control (C); Leuprolide (Leupr.); testosterone propiontae (T.P.); Leuprolide + testosterone propionate (Leupr. + T.P.)

measuring LC nuclear diameters. A 40% reduction of the nuclear diameter of LC (2.9 ± 0.07, $p<0.05$) for Leuprolide treated rats was measured when compared to nuclear diameter of control LC (4.9 ± 0.06, $p<0.05$). LC responded morphologically to hormone depletion, but this effect may not be due strictly to the absence of testosterone. Leuprolide acts directly on LC since these cells have LHRH receptors and Leuprolide may trigger the synthesis of factor(s) that act in an autocrine fashion.

Somatic Cell Counts

Morphometric procedures (Matikainen et al., 1994; Gaytan et al., 1992; Myers et al., 1991; Abney et al., 1991; Teerds, et al., 1989; Russell et al., 1981) were conducted to obtain quantitative information about LC and MC numbers. Only nuclei with unaltered morphology were counted randomly by selected interstitial areas of cross sections created by three adjacent seminiferous tubules. There is no significant difference in the relative numbers of SC in the Leuprolide (1931 ± 75.2, $p<0.05$), testosterone propionate (2040 ± 45.8, $p<0.05$), and Leuprolide + testosterone propionate (2026 ± 47.7, $p<0.05$) treatment groups when compared to control (1997 ± 130.4, $p<0.05$) (Figure 12).

Treated groups are significantly different from controls in the numbers of LC and MC. The Leuprolide treated group shows a 50% (432 ± 71.2, $p<0.05$) and 40% (133 ± 11.1, $p<0.05$) reduction of LC and MC numbers respectively when compared to those of the control group (878 ± 38.8, $p<0.05$; 228 ± 10.6, $p<0.05$). There is some maintenance of the numbers of Leydig cells

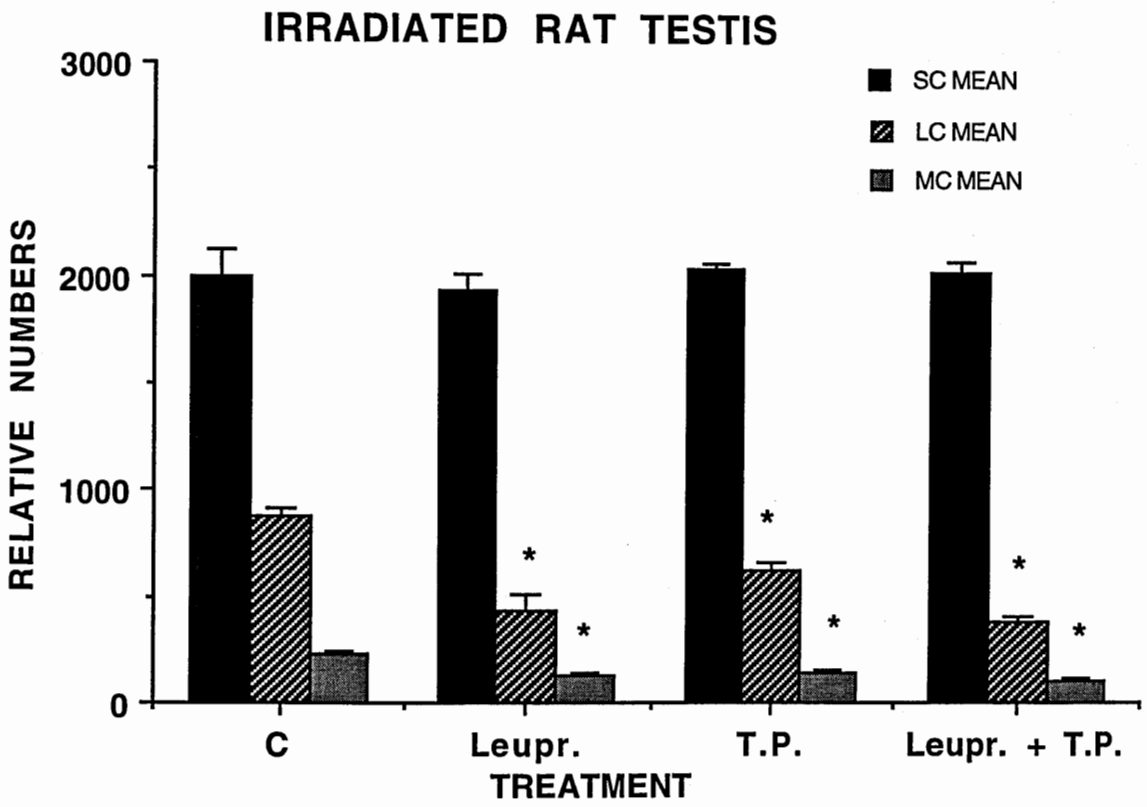


Figure 12. Relative numbers of somatic cells: Sertoli cells (SC), Leydig cells (LC), and myoid cells (MC) in the irradiated rat testis under different treatments

* Marks significant differences ($p<0.05$, Tukey test) of Leuprolide (leupr.), testosterone propionate (T.P.), Leuprolide + testosterone propionate (leupr. + T.P.) groups when compared to the control group (C). Using a light microscope (40X objective) SC and LC were easily identified and counted. Only nuclei with unaltered morphology were counted. To count MC it was necessary to use oil immersion (100X objective), since these cells were not visible at lower magnification.

(627± 34.7, $p<0.05$) and MC (141 ± 6.8, $p<0.05$) in the testosterone propionate treated group. These cells were not sustained at the numbers of the control group. Likewise, in the combination group of Leuprolide plus testosterone propionate, the reduction of LC (376 ± 26.4, $p<0.05$) and MC (104 ± 6.5, $p<0.05$) numbers is evident when compared to controls. LC were easy to identify and count using a 40X objective. MC are very small and can easily be confused with epithelial cells in the IS. Therefore, oil immersion with a 100X objective was used to count MC.

Radioimmunoassay for Serum Hormones

Constantly maintained levels of Leuprolide and replacement of androgen has substantial effects on serum concentrations (ng/ml) of testosterone (T), luteinizing hormone (LH), and follicle stimulating hormone (FSH) (Table 4). Testosterone levels of the Leuprolide treated group decreased 70% (0.9 ± 0.7, $p<0.05$) when compared to values of the control group (3.1 ± 0.6, $p<0.05$). Since testosterone propionate was administered exogenously to groups treated with this androgen with or without Leuprolide, a supranormal level of this hormone in the serum was expected. These two groups are not considered statistically valid for comparison. However, these values are important in establishing that the10 fold to 20 fold excess androgen in the gonad was replaced exogenously. The T level obtained from the irradiated control serum was in the high normal physiological range,

Table 4

Serum concentration (ng/ml) of testosterone (T), luteinizing hormone (LH), and follicle stimulating hormone (FSH) in irradiated rats under different treatments

Treatment	n	T	LH	FSH
Control	(6)	3.1 ± 0.6	4.2 ± 0.2	68.4 ± 11.8
Leupr.	(6)	0.9 ± 0.7*	2.0 ± 0.2*	12.7 ± 0.5*
T.P.	(6)	54.6 ± 30.3	2.1 ± 0.2*	132.2 ± 41.2
Leupr.+T.P.	(6)	42.5 ± 30.8	1.6 ± 0.3*	35.5 ± 6.7

* Marks significant differences ($p<0.05$, Tukey test) when compared to controls.
n = number of animals per group

Levels of testosterone (T), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined in rat serum using radioimmunassay (RIA) kits. Higher levels of testosterone propionate were expected in groups treated with this hormone. These two groups were not considered statistically valid for comparison.

suggesting that irradiation did not adversely affect the normal negative feedback of this hormone.

LH levels decreased in the Leuprolide ($p<0.05$; 48%) in the testosterone alone ($p<0.05$; 50%) and in the Leuprolide + testosterone treated group ($p<0.05$; 38%) respectively, when compared to the control group ($p<0.05$; 100%). Supranormal levels of testosterone were found in the testosterone treated groups with and without Leuprolide and suppressed the levels of LH in these two groups, suggesting inhibition of LH on pituitary cells by negative feedback. Testosterone and its metabolites also act on the central nervous system to slow the hypothalamic pulse generator and consequently decrease the frequency of the LH pulsatile mechanism (Griffin and Ojeda, 1988).
In the Leuprolide treated groups, the low serum level of LH was expected in spite of the absence of T, since this agonist superseded any pulsatile release of GnRH from the hypothalamus, which in turn, prevents secretion of LH. The levels of T and LH in the control group suggest that a coordinated hypothalamic-pituitary-testicular interrelationship was maintained, which makes this an appropriate group for comparison with other treated groups.

FSH concentration (ng/ml) in the Leuprolide treated group decreased to 19% (12.7 ± 0.5, $p<0.05$) of controls. In the testosterone treated group FSH increased to 94% (132.2 ± 41.2, $p<0.05$) greater than control (68.4 ± 11.8, $p<0.05$).

Incorporation of [^{35}S] Methionine /Cysteine into Proteins

Testes tissue was labeled in *vitro* with [^{35}S] methionine and cysteine and the incorporation rate into proteins in the testicular tissue pellet and in the labeled medium was recorded to elucidate whether the different treatments had an effect in the incorporation rate. Incorporation (counts per minute per μg of protein (cpm/μg)) of [^{35}S] methionine/cysteine into proteins was much the same for both the testes tissue and incubation media for each treatment group (Table 5). Thus, the incorporation of amino acids into protein was not affected by any treatment.

SDS Polyacrylamide Gel Electrophoresis and Autoradiography

In an attempt to establish specific changes in the testes following long term hormonal treatments, tissue proteins were labeled by *in vitro* incubation in medium containing [^{35}S] methionine/cysteine with reduced serum levels. Proteins from testicular tissue and secreted proteins (medium) were separated by size on 10% polyacrylamide gels containing SDS (Figure 13). No major changes in stained proteins profiles from tissue or medium was evident in any of the treatment groups. The protein patterns in the testicular tissue pellet and medium were reproducible from one testes preparation to the next which is supportive of having a consistent cell population in the irradiated testes.

Autoradiographic analysis (Figure 14) reveals that [^{35}S] methionine/cysteine was incorporated into a wide range of proteins. At least 12

Table 5

Incorporation of [^{35}S] methionine/cysteine intoTCA precipitable protein of irradiated rat testes

Treatment	n	cpm/µg protein		% of control	
		Tissue	Medium	Tissue	Medium
Control	(6)	4821 ± 782	1133 ± 130	100	100
Leupr.	(6)	6741 ± 133	1015 ± 225	140	90
T.P.	(6)	3434 ± 529	1191 ± 109	71	105
Leupr.+ T.P.	(6)	4521 ± 799	1046 ± 93	94	92

The values shown are the Mean ± SE
n= Number of animals per group.

Tissue was incubated for 5 h at 34°C, while being gassed with 5%CO_2 and 95%O_2. The incorporation of radioactivity into proteins was determined by TCA precipitation. No significant differences were observed in cpm/ug protein values among any treated group ($p<0.05$). No significant differences were observed in %cpm/ug protein values among any treated group ($p<0.05$).

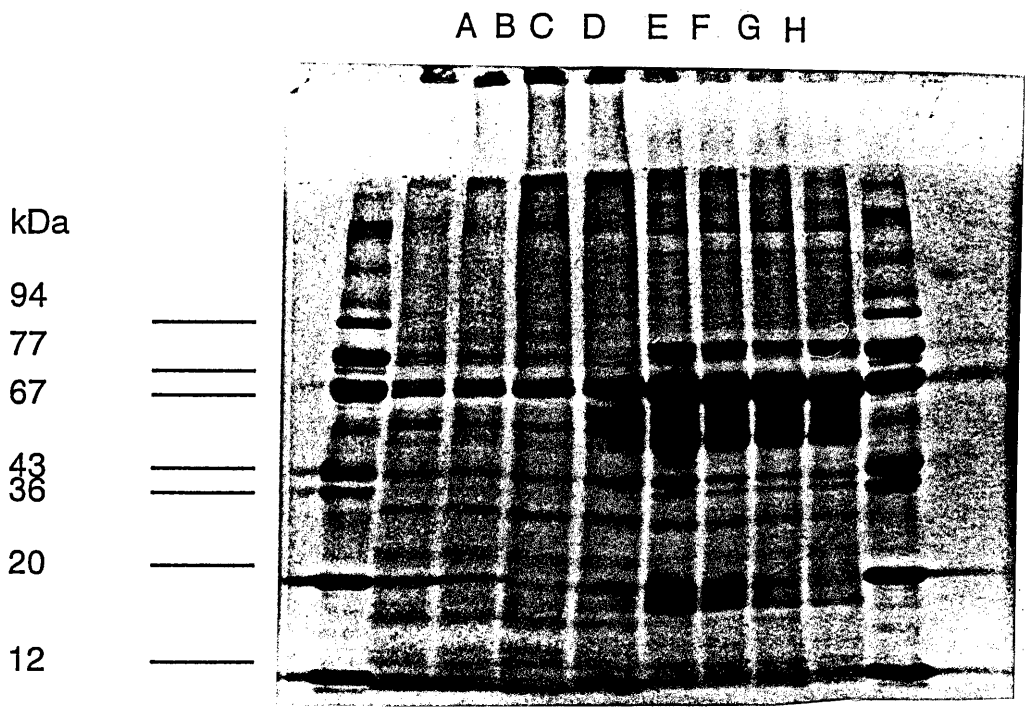


Figure 13. Stained protein profile from SDS-polyacrylamide gel (10%) of testicular tissue and proteins from the incubation medium for labeling protein

Proteins of testicular tissue are in lanes A to D, and proteins from the incubation medium are lanes E to H. Treatments groups are: Controls (A, E); Leuprolide (B, F); testosterone propionate (C, G); and Leuprolide + testosterone propionate (D, H). Numbers at the left lanes are the molecular weight standards shown at the left and right. Stained proteins profiles are very similar from different groups. Most of the proteins shown in the medium are serum proteins.

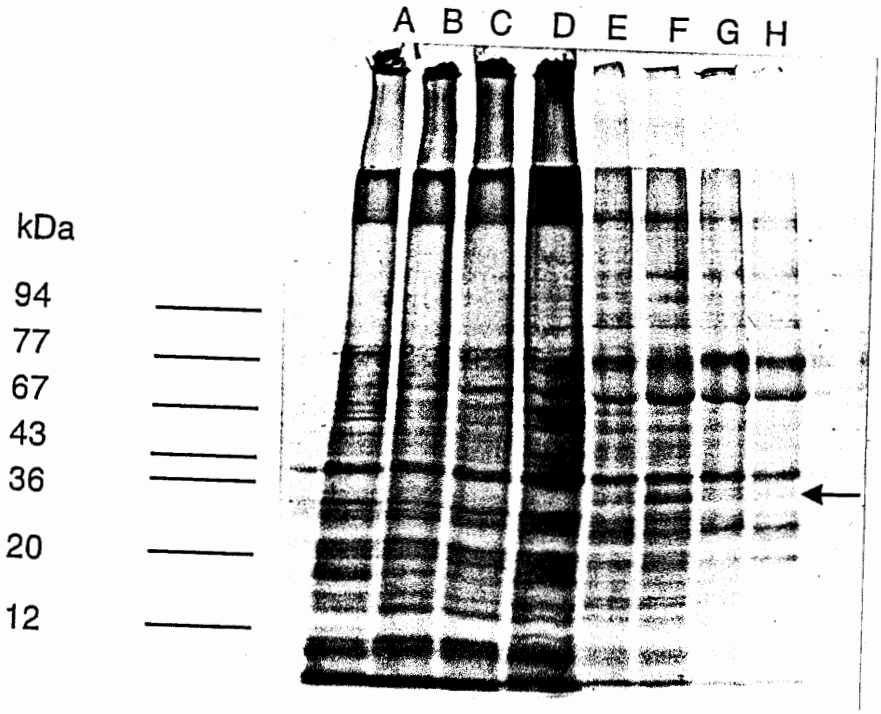


Figure 14. Autoradiograph of [^{35}S] labeled protein from testicular tissue (A-D) and proteins secreted or released into medium (E-H) during *in vitro* incubation

Autoradiography was performed with X-omat X-AR 5 film exposed for 6 days. Treatments groups are: Control (A, E); Leuprolide (B, F); testosterone propionate (C, G); and Leuprolide + testosterone propionate (D, H). The most readily detected band in the medium corresponds to a 36 kDa protein that is fairly abundant in the Leuprolide treated group (shown by an arrow). This band was reproducible at different intensities in all Leuprolide treated samples.

proteins were common in both testis tissue pellet and medium, with the following kDa: 14, 16, 18, 20, 24, 32, 43, 49, 55, 61, 67, 77. These proteins were present in all treated groups. The accumulation of these proteins in the tissue pellet may be due to the abundance of their synthesis coupled with their slow rate of turn over. There are three proteins whose presence in the testicular tissue was highly reproducible (25 kDa, 28 kDa, and 70 kDa). Occasionally proteins of this size were also found in the medium protein profile of all the testis preparations.

The most interesting band present in the media corresponds to a 36 kDa protein. Unlike the three proteins found only in the pellet and common to all treated groups, this protein was unique, because its synthesis and secretion appeared to increase in the presence of Leuprolide. This may indicate that either the absence of testosterone is permissive to its secretion or Leuprolide itself may trigger its synthesis. In the medium, there were apparent changes also in minor proteins (judging by their intensity in the autoradiograph) in all treated groups (such as 58, 63, and 85 kDa). Similarly, two bands of 94 and 116 kDa are present in the medium of Leuprolide + testosterone propionate group (Leupr.+T.P.), but only the former is present in the Leuprolide treated group (Leupr.) and the latter in the testosterone treated group (T.P.). Also, the presence of these two bands was not highly reproducible in all samples of the same treatment, which suggests that they may be products of artifacts. Judging from their molecular weight, these bands could also be secretory products of MC which are known to secrete proteins with high molecular weights.

Nevertheless, since changes in these minor proteins were not consistent, they were not pursued. Some bands were evident in the commassie gel but not in the autoradiograph. These proteins probably originated as a product of some cells from the testis prior to incubation.

Density Trace of Autoradiogram using an Image Analyzer

Autoradiographic profiles of pellet tissue proteins (Figure 15 A) of the irradiated rat testis showed no major relative difference among the treated groups. In the medium protein profile (Figure 15 B), a 36 kDa band is very visible and is shown by an arrow. This band was reproducible at different intensities in all Leuprolide treated samples.

Quantitative Comparison of Labelled Protein

To quantify the relative amount of proteins present in the testis tissue pellet and in the medium, the Relative Optical Density (ROD) values of the bands in the autoradiograms were determined.

The ROD value is defined as a unit of density, and can be expressed by the equation:

$$\text{ROD} = \log_{10} \frac{\text{maximum number of gray levels}}{\text{observed gray levels}}$$

The greater the relative concentration of the sample in each band, the greater the intensity of the band will be, and the intensity in turn will correlate with the ROD value of each band.

Figure 15 A. Density trace of autoradiogram of testis tissue pellet shown in Figure 14

The autoradiogram was subjected to image analysis to trace each band present in each treatment group. Each peak identifies a protein or protein component distributed by size as shown in the bands of the autoradiogram in Figure 14 (Lanes A to D). No major changes were observed in any treated group: Control (1); Leuprolide (2); testosterone propionate (3); and Leuprolide + testosterone propionate (4). Therefore, the effect of testosterone, is not restricted to any specific protein because all treated groups have a similar protein profile.

Figure 15 A

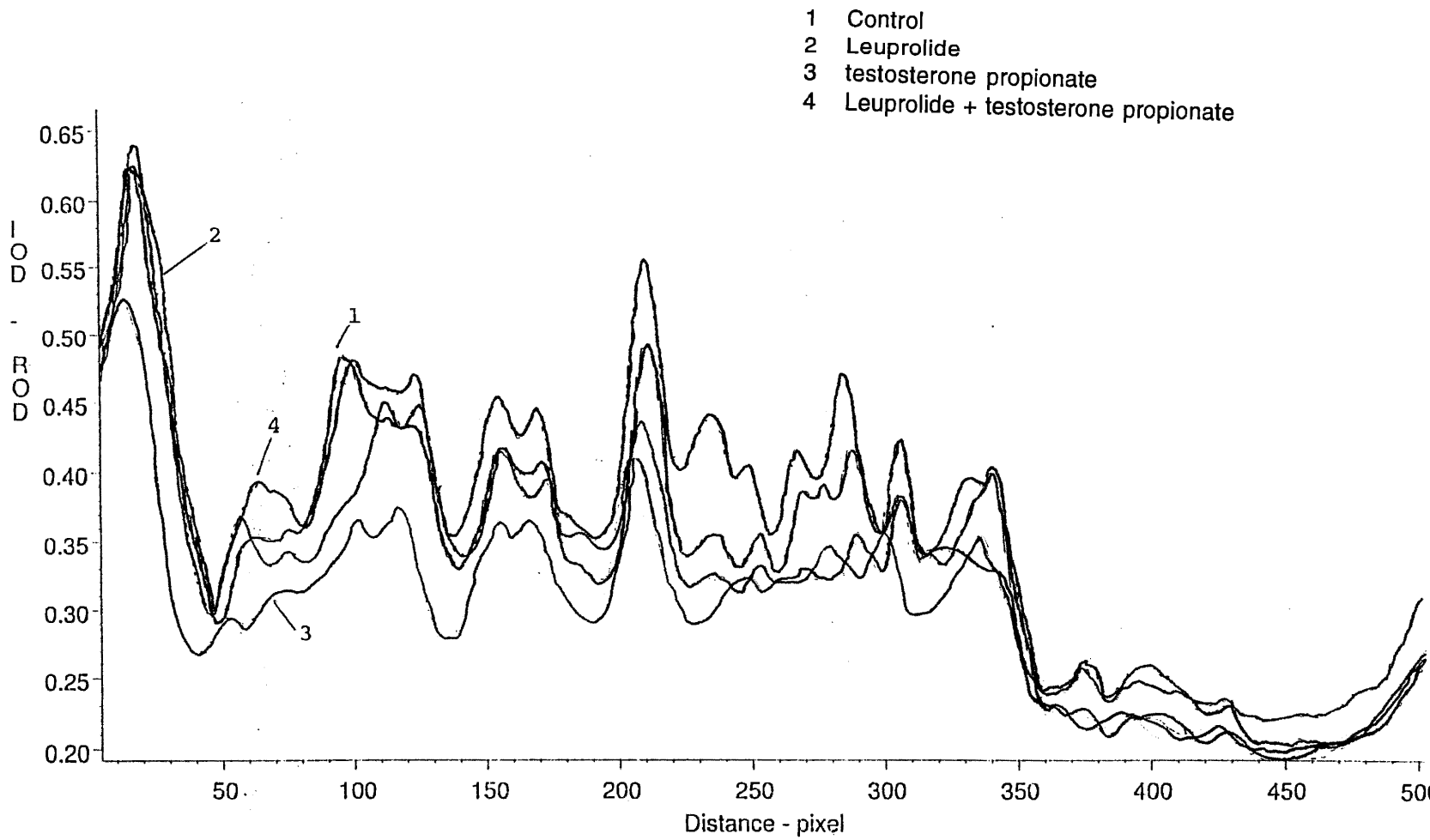
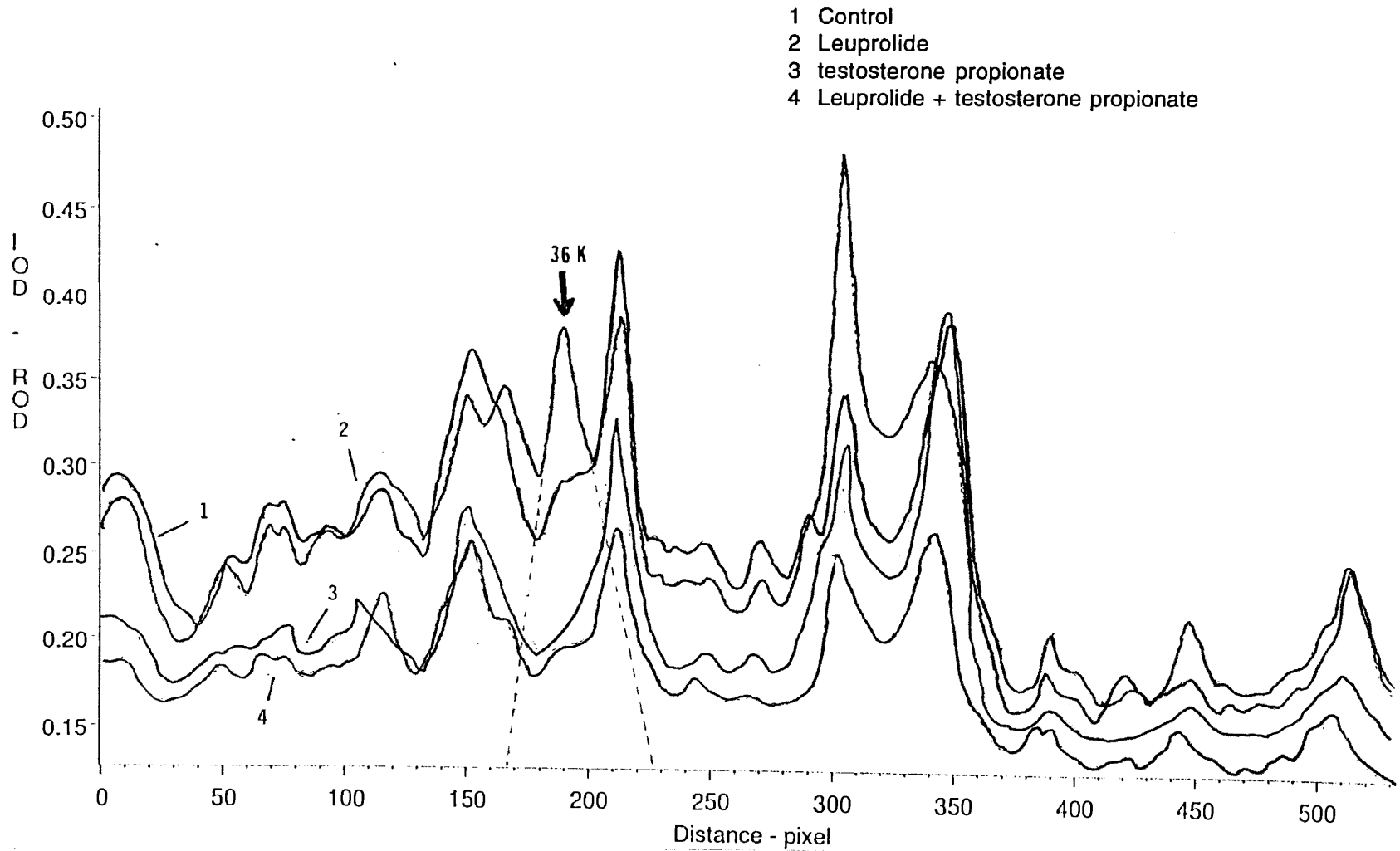

1 Control
2 Leuprolide
3 testosterone propionate
4 Leuprolide + testosterone propionate
IOD - ROD
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
50
100
150
200
250
300
350
400
450
500
Distance - pixel

Figure 15 B. Trace of autoradiogram of rat testis protein secreted into the medium shown in Figure 14

The autoradiogram were subjected to image analysis and each peak identifies a protein or protein component distributed by size as shown in the bands of the autoradiogram in Figure 14 (Lanes E to H). Treatments are: Control (1); Leuprolide (2); testosterone propionate (3); and Leuprolide + testosterone propionate (4). The most distinctive difference as a reproducible peak corresponds to a 36 kDa protein. Secretion of the protein was enhanced in the absence of testosterone in the Leuprolide treated group.

Figure 15 B


1 Control
2 Leuprolide
3 testosterone propionate
4 Leuprolide + testosterone propionate
36 K
IOD - ROD
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0
50
100
150
200
250
300
350
400
450
500
Distance - pixel

The ROD and percentage values were determined from six autoradiograms of each treated group, and the values are expressed as a percentage of the total ROD value in the same lane of the autoradiogram (Table 6 and Table 7).

The ROD and percentage values (Table 6) of each reproducible band present in the testicular tissue revealed no significant difference on the ROD values of any band present in any treated group. That is no difference were observed with androgen withdrawal (Leupr.) and androgen maintenance (T.P.) or in a combination (Leupr. + T.P.) treated groups when compared to the control group (C). Overall, the ROD and percentage value of all bands present in the autoradiogram of the rat testis pellet ranged between 0.1 ROD -0.4 ROD; and 1.4% and 8.2%. The lowest percentage (ranges between 1.4% and 2.2%) corresponded to a protein of 55 kDa molecular weight in the testosterone treated group.

However, a band of 14 kDa was noteworthy because of the slight increase of the intensity (0.4 ROD) in the Leuprolide treated group when compared to that of the control group and to other treated groups (0.3 ROD). This result suggests that the amount of synthesis of this protein was slightly higher (0.1 ROD value) than in the control group and in the other treated groups. The percentage value of this 14 kDa protein ranged between 7.9% and 8.2% of the total ROD value of each treatment group, reflecting the presence of this protein as a major component in each treated group, including the control group.

Table 6

Relative optical density (ROD) and percentage values of autoradiographic bands from irradiated rat testis tissue

Band No	MW (kDa)	Treatments							
		Control (n=5)		Leuprolide (n=5)		Testosterone Propionate (n=5)		Leuprolide + Testosterone Propionate (n=5)	
		ROD	%	ROD	%	ROD	%	ROD	%
1	14	0.3	7.9	0.4	8.0	0.3	8.2	0.3	8.0
2	16	0.2	3.3	0.2	3.0	0.1	2.6	0.3	2.4
3	18	0.2	1.5	0.2	2.6	0.2	2.5	0.2	2.2
4	20	0.2	3.6	0.3	3.7	0.2	3.2	0.2	3.1
5	24	0.2	3.2	0.3	4.0	0.2	2.9	0.2	2.5
6	25	0.2	2.8	0.2	3.4	0.3	2.9	0.3	3.3
7	28	0.2	3.4	0.3	3.7	0.3	3.9	0.3	3.8
8	32	0.2	3.3	0.3	2.9	0.2	3.9	0.3	3.7
9	43	0.2	6.1	0.3	5.5	0.3	6.0	0.3	6.0
10	49	0.2	2.1	0.4	2.3	0.3	2.2	0.3	3.0
11	55	0.1	2.2	0.2	2.3	0.2	1.4	0.2	1.8
12	61	0.3	2.3	0.2	2.4	0.1	2.2	0.2	2.7
13	67	0.2	2.7	0.2	3.0	0.2	3.4	0.2	3.6
14	70	0.2	2.2	0.2	2.1	0.2	3.6	0.2	2.6
15	77	0.2	3.6	0.2	3.5	0.2	3.6	0.2	3.8

Note: Only reproducible bands found in each autoradiograph were considered
MW (Estimated Molecular weight in kiloDaltons (kDa)
n= Number of animals.

Table 7

Relative optical density (ROD) and percentage values of autoradiographic bands from irradiated rat testis tissue protein secreted into the medium

Band No	MW (kDa)	Treatments							
		Control (n=5)		Leuprolide (n=5)		Testosterone Propionate (n=5)		Leuprolide + Testosterone Propionate (n=5)	
		ROD	%	ROD	%	ROD	%	ROD	%
1	14	0.1	5.1	0.1	4.3	0.1	4.0	0.1	2.4
2	16	0.1	3.4	0.1	3.4	0.1	3.1	0.1	2.8
3	18	0.1	2.8	0.1	2.3	0.1	2.3	0.1	3.4
4	20	0.1	2.2	0.1	2.2	NRB		NRB	
5	24	0.1	3.6	0.1	4.7	0.1	3.7	0.2	3.5
6	32	0.1	9.5	0.2	7.4	0.1	8.0	0.1	6.2
7	36	NRB		0.2	5.5	0.1	3.0	0.1	3.7
8	43	0.2	6.5	0.1	7.3	0.1	7.5	0.1	7.6
9	49	0.1	2.0	0.1	2.5	NRB		0.1	2.4
10	55	0.1	2.2	0.1	2.1	NRB		0.1	1.9
11	61	0.1	2.2	NRB		NRB		NRB	
12	67	0.1	4.3	0.2	6.0	0.1	5.3	0.1	5.1
13	77	0.1	7.1	0.2	8.2	0.2	10.1	0.1	7.7
14	94	NRB		0.1	1.6	NRB		0.02	3.0
15	116	NRB		NRB		0.1	3.1	0.03	2.9

MW (Estimated Molecular weight in kiloDaltons (kDa)
n= Number of animals.
NRB = Not reproducible in all samples of the same treatment

The ROD values of 25 kDa and 28 kDa bands shown in the testis tissue, but not in the medium, were not different from the values obtained for other bands when compared to the control group. The ROD value of the 25 kDa protein in the testosterone plus or minus Leuprolide was 0.3 ROD, which was a 0.1 ROD higher with respect to the control and the Leuprolide treated groups. The ROD value of the 28 kDa band, on the other hand, was the same for each treated group (0.3 ROD). This value represented an increase of 0.1 ROD with respect to the control group (0.2 ROD). The percentage value of these two proteins represented no major changes in any treated group with respect to the control group (range between 2.8 % and 3.9 %).

Likewise, the ROD value (0.3 ROD) of a 43 kDa did not differ in any treated group from the control values (0.2 ROD) but it showed a slight increase in its intensity value (0.1 ROD) from the irradiated control group. The percentage range of the total ROD of this band was between 5.5% and 6.1% which showed that this protein made up a considerable amount of the total synthesized protein in each treated group including that of the control group.

The bands present in medium protein profile were not as intense as those of the pellet testes tissue. Their ROD values were between 0.1 and 0.2, which was 0.2 ROD less than the values obtained in the tissue pellet autoradiogram and their percentage values ranges between 1.6% and 10.1% (Table 7).

There were three bands (32 kDa, 43 kDa, and 77 kDa) in all treated groups that showed high percentage values (range between 6.2% and 10.1%)

with similar values to those of the control group, showing that these proteins may represent a significant percentage of the total secreted protein in each group.

A 36 kDa protein which intensity increased in the Leuprolide treated group has a ROD value of 0.2 higher than that obtained from other treated groups (0.1 ROD) and a percentage value of almost 6%. The uniqueness of this 36 kDa molecular weight protein was that it was well defined and reproducible in the Leuprolide treated group. Occasionally, this 36 kDa band was shown in the control group. Therefore its ROD value could not be determined in this group and it was represented as a non reproducible band (NRB). A diffused band with a similar molecular weight (36 kDa) was observed in the testosterone with or without Leuprolide, whose ROD was 0.1 and their percentage value was 3.7% and 3.0% respectively.

There were two other bands (32 kDa and 67 kDa) present in the Leuprolide treated group, whose ROD value of 0.2 was higher when compared to the control group and to other treated groups (0.1 ROD). Their percentage values were between 6.2% and 9.5%; 4.3% and 6% respectively.

Some protein bands from the medium were not reproducibly found or their intensity was not high enough to obtain their ROD value, therefore; these bands were also categorized as non- reproducible bands (NRB).

Since secretory proteins do not generally accumulate in the cell under normal circumstances, but rather are released into the medium, these proteins comprise a very small percentage of the proteins within the cells. No significant

differences were observed in the apparent abundance of individual proteins, but generally the same proteins were present in the same amounts.

The testosterone effect was not specific to any protein present either in the pellet or the medium since no drastic changes in ROD values were observed upon the administration of testosterone propionate. No protein was found to be under androgenic control.

CHAPTER IV

DISCUSSION

The present study demonstrated that Leydig cell morphology was altered by the Leuprolide treatment. It is presently believed that Leuprolide interferes with the functioning of the hypothalamic-hypophyseal-pituitary-gonadal axis, but several lines of evidence obtained in this study, indicated a direct effect of Leuprolide on the morphological changes observed in the Leydig cells. In addition, Leuprolide seemed to directly influence changes in protein profile. The presence of a 36 kDa protein in the Leuprolide-treated group suggested that this is a product of a response of Leydig cells to Leuprolide administration. The expected maintenance effect on somatic cells by exogenous testosterone was not observed in this study.

Leuprolide administration with or without testosterone propionate did not affect body growth; the animals continued growing during the experimental treatment in all treated groups. Conversely, a 53% and 52% reduction in testis weight with and without tunica albuginea, respectively, in the Leuprolide - treated group was observed, when compared to the controls. Testis weights with the tunica albuginea was measured, because it gives a more reliable weight which can be used to judge the reliability of the weight assayed without the tunica albuginea. A reduction in testis weight (with or without tunica albuginea, respectively) of 32% and 30%, was also observed in the

testosterone alone group and in the combination with Leuprolide treated group (46% and 44% respectively). This result was interpreted as an absence of maintenance by androgen on testicular weight. These findings are in agreement with the work of Kholo and Huhtaniemi (1989) who showed that the GnRH agonist in normal rats severely decreased testis weight without affecting body weight, demonstrating a lack of correlation between body growth and testicular weight.

Restoration of testicular weight was shown in normal rat testes treated with GnRH agonist after testosterone with or without FSH administration (Itoh et al., 1994) and partial maintenance of testicular weight from hypophysectomized irradiated rats after EDS with or without testosterone administration (Mills, 1990). Collectively, results of these previous experiments are consistent with the suggestion that testosterone had a direct and primary rather than secondary, effect on testis weight. However, results of the present study suggest that Leuprolide rather than testosterone may have a direct effect on rat testes weight. Testicular weight in GnRH agonist treated rats was partially restored following separate administration of FSH or testosterone, but it was completely restored after combined treatment with both FSH and testosterone (Ganguly et al., 1994).

The morphometric analysis in this study revealed a reduction of seminiferous tubule (ST) and Leydig cell (LC) diameters in the Leuprolide-treated animals. This observation correlates with the reduction of the testicular tissue to 50% of control.

The observed reduction of ST diameter was probably due to a decrease in luminal area, although this was not measured. Setchell and Sharpe (1981) postulated that one of the effects of GnRH agonist is a reduction in Sertoli cell (SC) fluid production and/or an alteration in the rate of flow. Since SC are the major cell type to secrete fluid into the tubular lumen (Jegou, 1992), one of the earliest effects of Leuprolide may be to interfere with the secretory activity of these cells.

The interstitial space (IS) in the Leuprolide treated testes was reduced in size and was stained intensely with PAS (specific for monosaccharide, polysaccharide residues, glucose, galactose, or sialic acid (Kiernan, 1990)). The size of the IS could result from the reduction of the interstitial space coupled with the decrease in the volume of interstitial fluid. It is possible that only the interstitial area was reduced but a normal basal level of fluid was present. Results in this study have shown the severity of morphological changes in Leydig cells in the Leuprolide-treated groups. Various means to lower pituitary hormones affecting Leydig cells are described in the literature, such as the neutralization of pituitary hormones by specific antibodies, or destruction of LC by chemicals. Russell et al. (1992) suggested that these compounds act by similar mechanisms, and can be used for comparison of results obtained in this study.

The present study, LC showed a marked morphological change and a reduction of nuclear diameter with Leuprolide treatment. The administration of exogenous testosterone did not maintain these parameters. Although the cells

were altered and reduced in size, their identification was not difficult having retained many of the characteristics observed in the irradiated controls. The mechanisms by which LC regress remain uncertain (Catt and Dafau, 1978) and it is believed that these effects are due to the absence of LH. LC nuclear size and structure were affected in normal rats treated with a GnRH antagonist but maintained their original nuclear shapes and mean nuclear diameters following testosterone administration (Misro et al., 1993). By comparing the results of Misro et al. (1993) with the results of the present study, it can be assessed that LH may be only partially responsible for the alteration of LC morphology and reduction of the diameter size. In agreement with this idea, normal LC morphology and nuclear diameter were not maintained following testosterone administration to LH depleted animals (testosterone alone treated group) or testosterone in combination with Leuprolide.

The maintenance of SC nuclei normality may be due to either the failure to disrupt the ST environment, or to the intrinsic ability of these cells to resist ST changes. The latter possibility is more likely, since Galdieri et al., (1981) have demonstrated that SC were able to resist osmolarity changes in the environment by compartmentalizing water in the cavities of the endoplasmic reticulum. The ability of these cells to resist such changes could be due to their location between two compartments of different osmolarities at the border of the seminiferous epithelium. The numbers of SC were not affected by any of the treatments which correlated with the lack of changes in their morphology. This result is in agreement with Rowlet and Heller (1971) who postulated that the

number of SC remains constant, even under severe treatments. The average number of SC per tubule was between 15 and 16 in all treatment groups used in this study, which is in close agreement with the results of Bustos-Obregon (1970) who showed that the average of SC numbers was about 12.2 for each ST. Somatic cell identification for stereological analysis was based on the observation of their morphological patterns described by Gaytan et al. (1994) and others. Bustos-Obregon (1970) considered nucleolar count to be a more reliable system for stereological analysis because of the polymorphism of SC cytoplasmic outlines. Nearly all SC had a single nucleolus in all the treated groups. The increase in SC nucleolar diameter size by 31% in the Leuprolide-treated group when compared to the control was unexpected. The volume of the nucleolus is a reflection of its biosynthetic activity and can be up to 25% of the total nuclear volume in cells that are making unusually large amounts of proteins (Alberts et al., 1989). This reflects the demand for increase numbers of ribosomes. Therefore the modification of the SC nucleoli could be interpreted as changes in SC synthetic activity due to either a direct or indirect effect of Leuprolide on the transcription and translation.

LC and MC numbers were reduced in the Leuprolide treated group by 50% and 40%, respectively, when compared to control values. Similar results were obtained by Kerr et al. (1987) after treatment of rats with a GnRH antagonist. Their report showed a reduction in the average numbers of LC, but significant changes were not seen in the total number of cells present in the interstitium. Even though the present study did not quantify the total number of

cells observed in the interstitium, numerous unidentified interstitial cells in addition to LC were observed. The presence of numerous cells other than LC was also reported by Drobeck and Coulston (1962), although they considered their result to be a consequence of change in volume of the IS due to the atrophy of the ST. Iturriza and Irusta (1969), on the other hand, have suggested that the IS hyperplasia was due to the increase in the number of cells other than LC. Likewise, the presence of new and extensive proliferation of undifferentiated stem cells in the IS occurs after treatment with cytotoxic drugs (Kerr et al., 1987) or EDS (Bartlett et al., 1986; Teerds et al., 1989b; Teerds et al., 1988a). These authors could not clearly identify the new cells but they suggested that the action of growth factors could account for their formation.

The normal number of LC in the present study was not maintained by testosterone treatment, contrary to the results of Misro et al. (1991). Evidently, testosterone is not the sole factor responsible for LC number in the present study but there was no clear indication that LH alone was exclusively necessary for LC maintenance.

Kerr and Donachie (1986) favored the idea that any disruption of the seminiferous tubules (as occurred in the Leuprolide treated group) is capable of modifying LC morphology and function. Paracrine stimulation and regeneration of Leydig cells has been hypothesized by Kerr and Sharpe (1985) in the studies of Leydig cells after treatment with EDS. It was concluded from

their studies that LH is not necessary for the stimulation of the proliferation of the possible LC precursor.

The morphometric and stereological changes of somatic cells in this study are undoubtedly a product of a general imbalance generated through the duration of the experiment. The literature reports on mammalian systems often vary considerably from laboratory to laboratory (Ghosh et al., 1992). This observation makes it more difficult to discover mechanisms behind the results obtained in any morphometric studies. For example, several of the testicular parameters, including testis weight and tubular and LC diameters, all correlated positively with the numbers of LC, SC, and MC; however, body weight did not correlate with these values. A number of significant morphological changes was observed in the testicular tissue and somatic cells in the Leuprolide treated testes. The complex process of such changes must have involved modifications in cell shape and structure and in several structural connections between SC, MC, LC, and other interstitial cells. This, is turn, could lead to the alteration of the interactions between the testes and other accessory sexual organs.

Results from this study did indicate that Leuprolide may have one or more targets among the somatic cells in the male reproductive system. These cells may have undergone small modifications with numerous morphological and functional consequences. This hypothesis appears to be supported by the finding of vacuolation in prostate cancer cells induced directly by Leuprolide (Muir et al.,1994).

It is worthwhile to mention that two of the six animals in these experiments developed testis tumors following Leuprolide with or without testosterone propionate treatment. Almost 60% of the ST present in the testicular tumors were severely affected. The tissue had deteriorated so badly that the tubules had the appearance of a uniform deep pink mass as a result of heavy staining with the PAS stain.

Serum hormone levels were also measured. Leuprolide was effective in reducing testosterone levels (T) by 70% when compared to control values. There was some testosterone synthesis with about half of the animals having testosterone in the serum, although all Leuprolide treated animals had a reduced testis weight. Interestingly, the levels of LH in the same animals were very low. This suggests that Leuprolide may have a direct effect on testis tissue in addition to blocking the production of LH from the pituitary gonadotrophs. Kholo and Huhtaniemi (1989) have shown a high concentration of T in GnRH agonist treated animals. They suggested that only a small fraction of LH receptors is needed for the maintenance of steroidogenesis. The loss of LH receptors after GnRH agonist treatment is probably due to receptor down regulation and /or occupancy by the heightened LH secretion during the brief stimulatory period at the beginning of GnRH agonist treatment. Likewise, Misro et al. (1992), working with rats treated with GnRH antagonist, demonstrated that LH levels remained undetectable, while T levels were restored to near control levels.

LC steroidogenic activity *in vitro* and *in vivo* has been suggested to depend on LH (de Kretser, 1982). However, there is increasing evidence to suggest that intratesticular concentrations of testosterone are regulated by local factors which may emanate from the seminiferous tubules and be transferred across the basement membrane (Parvinen, 1982; Sharpe, 1983). In accordance with this suggestion, Leuprolide may have stimulated the production of these local factors in some animals and produced a higher concentrations of androgen in the serum.

In studies of LC structure and function smooth endoplasmic reticulum (SER) volume has been correlated with testosterone production (Misro et al., 1991). No effort was made in the present study to determine whether SER volume in LC correlates with androgen serum levels in animals treated with Leuprolide, in which plasma T levels were only partially reduced. Similarly, Ahmann et al. (1987) treated prostate cancer patients with GnRH agonist for 3 months and reported that during this period almost 7% of the patients, had T levels exceeding the level of castration. T elevation was not accompanied by increases in either FSH of LH, nor were these changes associated with any adverse effects.

Testosterone has been implicated in the regulation of pituitary responsiveness to LHRH, via effects on LHRH-receptors, suggesting its importance in the regulation of gonadal LHRH agonist receptors (Sharpe and Fraser, 1980). Previous experiments failed to suppress sperm production after

T supplementation (Pavlov et al.,1988 and Behre et al.,1992) in normal rats treated with GnRH agonist.

GnRH agonist has different responses in males of different species and the basis for this divergence is not understood (D'Occhio and Aspden, 1996).

In the present study, supranormal administration of testosterone propionate was given to the testosterone alone treated group. This dose was necessary to mimic the androgen concentration inside the gonads, which is 10 to 20 times more concentrated than in the circulation. Zirkin et al. (1994) reported that the concentration of testosterone in the seminiferous tubule fluid of the rat testis is 50-60 ng/ml. The present results have shown that under testosterone maintenance, LH levels in the serum of rats decreased (due to GnRH inhibition by testosterone) but FSH serum levels increased. The physiological significance of the high level of FSH, however, is uncertain, although treatments that alter the T levels within the testis also frequently alter the volume of interstitial fluid and/or its rate of turnover (Sharpe, 1983). Bagatell et al. (1989) used supraphysiological doses of testosterone (200 mg/week) which suppressed gonadotropin secretion in contraceptive trials.

The higher levels of FSH found in this study may have several explanations: First, there is increasing evidence that gonadal peptides play an important role in the regulation of normal pituitary FSH biosynthesis (Steinberger and Steinberger, 1976; Christensen et al., 1994). The elevated FSH serum in this treated group could be due to the reduced availability of inhibin, a nonsteroidal inhibitor of FSH in normal testes. These changes could

be interpreted as a decrease in negative feedback arising from reduced inhibin secretion by SC. Similar decreases in inhibin have been documented by other investigators after cryptorchidism which alters the feedback loop that regulates FSH secretion via-nonsteroidal substances secreted by the ST (de Kretser and Robertson, 1989; Mc Lachlan et al., 1988). These authors have shown that the number of FSH receptors decreases after prolonged cryptorchidism. This result may represent down regulation of receptors resulting from increased FSH levels, or may be due to increased production of substances within the testes that are capable of preventing the binding of FSH to its receptors. A selective rise in plasma FSH has also been reported following a single injection of androgen or estrogen to immature female rats (Navqui and Johnson, 1969) and following busulphan treatment (Debeljuk et al., 1973).

This study cannot be used to determine if rats treated with T were less sensitive to the feedback effects of inhibin. However, such a possibility is strengthened by the suggestion of Christensen et al. (1994) that rats are less sensitive to the feedback effect of inhibin and the feedback effect of T may also partially mask the suppression of FSH by inhibin.

Roberts et al. (1989) have shown that endogenous levels of inhibin subunits in pituitary gonadotrophs increase after removal of rat ovaries; subsequent estrogen replacement therapy prevents this increase. This result raises the interesting possibility that gonadal factors modulate inhibin subunit production by pituitary gonadotrophs. A second explanation for the higher levels of FSH is that under the environmental condition created by the

testosterone treatment, androgen may also trigger the production of activin, which in turn, stimulates FSH secretion. *In vitro* and *in vivo* experiments have shown that testosterone increases the secretion of activin by pituitary cells (McLachlan et al., 1989), which stimulates FSH secretion even in the after of desensitization of GnRH (Schwall et al.,1988).

The diversity of testicular paracrine action necessitates control of inhibin and activin action on several levels, such as synthesis and secretion of proteins an expression and regulation of functional receptors (Moore et al., 1994). To date, two distinct binding proteins for inhibin and activin have been identified, both are expressed in the testis. Follistatin is a binding protein which suppresses pituitary FSH release by binding directly to activin (Nakamura et al., 1990) and with lesser affinity to inhibin (Shimonaka et al., 1991). In testis, inhibin and activin act as functional antagonists and are coexpressed (Moore et al., 1994). Thirdly, the excess T may trigger specific cells in the hypothalamus to release LHRH, which, in turn, may trigger specific pituitary gonadotrophs to stimulate the secretion and release of FSH. Therefore, the rate of LH (found in low concentration in serum) and FSH secretion may be differentially responsive to regulation, as was originally suggested by Swerdloff and Walsh (1973). It is likely that there is a physiologically important releasing factor for FSH in addition to GnRH. This was suggested by the failure of a complete suppression of serum levels of FSH after a total GnRH receptor blockade (Hall et al., 1990).

LH and FSH are heterodimers comprised of a hormone specific beta subunit and a common alpha subunit. Putative regulators of FSH were

suggested by Moore et al. (1994) and LH appears to be controlled primarily by negative feedback. However, the negative feedback inhibition by testicular hormones on FSH secretion is not completely understood.

In the present study, higher FSH serum levels (when compared to the Leuprolide alone treated group) were also observed in rats with combined GnRH agonist and testosterone treatment. These results raise the possibility that testosterone may be capable of supporting FSH synthesis/release *in vivo* under circumstances that affect the normal interrelation and function of the hypothalamus-pituitary axis. This, in turn, could explain pituitary unresponsiveness to hypothalamic GnRH.

The higher FSH levels observed in the serum of rat treated with T (with or without Leuprolide) is of reasonable concern for many laboratories that are investigating contraceptive strategies in men. If indeed T alone or in combination with a GnRH agonist triggers FSH production, the intended contraceptive environment would be hard to achieve.

Under normal physiological conditions, T has negative feedback effects directly at the pituitary and hypothalamus level (Bagatell et al., 1989) which means that the combined administration of GnRH agonist plus T will inhibit gonadotropin secretion by the separate actions of the agonist and T.

In the present study, information on the number of proteins synthesized and secreted *in vitro* following different treatments, was obtained as well as features of them including molecular weight (MW), hormonal dependency, and percentage of synthesis in both the testicular tissue and labeled medium.

Although, the cellular source of the proteins could not be determined, the possibility that germ cells are the source of these proteins can be eliminated. Abdullah et al. (1986) reported that SC can selectively internalize proteins from the medium if the medium is supplemented with fetal bovine serum, indicating that several approaches must be used to substantiate the claim that a particular somatic cell in the rat testes *in vivo* is the source of a particular protein (s). Most of the same proteins were found in the cells and in the medium. The presence of the proteins in the medium is an indication that these proteins may be secretory and their unequal distribution in the tissue pellet and medium indicates that this was not a result of cell lysis. The protein bands were numerous and more intense in the cells than in the medium, indicating some cellular storage of the secretory proteins.

Changes were observed in the overall protein profile of the proteins secreted into the medium. The presence of a 36 kDa band in the media of the Leuprolide-treated animals was very intriguing, not least because its synthesis and secretion appeared to be dependent either on the absence of T or on a direct effect of Leuprolide on the testes tissue. The second possibility is more likely, since a band of similar size was also seen in the combination treated group (Leuprolide plus testosterone propionate) and was less evident in the testosterone alone treated animals.

The changes in protein profile in rat testes treated with the GnRH agonist in this study are not in agreement with the results of Verhoeven et al. (1995), who reported no changes in protein profile. One possibility for the discrepancy

may reside in the duration of the GnRH agonist treatment, since Verhoeven treated his experimental rats for 3 weeks and the duration in the present work was 6 weeks. An effect on somatic cells might be expected to be smaller after shorter treatment duration. Similarly, differences between the culture conditions of the two studies should be taken into consideration. Testis tissue was cultured for 3 days in Verhoeven's study; this may have resulted in a greater percentage of cell death during the incubation period. In comparison, the present study used only 5 hours incubation. Previous kinetic studies performed in our laboratory (unpublished data) have shown this period to be optimal labeling and for higher incorporation of amino acids into proteins.

Superactive GnRH agonists inhibit reproductive function by initially causing pituitary hyperstimulation followed by a decline in LH and FSH secretion as the gonadotrophs become desensitized (Cooper and Cernovsky, 1994) resulting in a 50% reduction of LH receptor content (Kholo and Huhtaniemi, 1989). The decline of LH levels in circulation stops the gonadal production of testosterone by LC. Some evidence for the direct effect of Leuprolide in the morphological changes of somatic cells was shown in this study. This hypothesis was supported by the observation that testes tissue regressed even in the presence of testosterone in some Leuprolide treated animals. The possibility of a direct effect of GnRH agonist in the testes has been previously hypothesized by few authors. A support for this concept was demonstrated by the presence of specific receptors for GnRH agonist on interstitial cells in vivo (Clayton et al.,1980) and by reports of a reduction of both

LH and hCG receptors in Leydig cells in hypophysectomized male rats (Hsueh and Erickson, 1979). Neither study showed what the possible results of the effects would be. The present study may be the first to demonstrate morphological alterations of somatic cells along with changes in protein profile that result from direct action of Leuprolide on testicular tissue.

Lefebre et al (1980) hypothesized and Sharpe and Fraser (1980) demonstrated that LC from adult rat testes contain high affinity binding sites specific for LHRH and its agonists. In light of these and those of the present study, the 36 kDa protein may be a product of LC that is synthesized as a defense mechanism. Sharpe (1983) identified fluid factors that are secreted only when seminiferous tubules were damaged, and it was suggested that this may indicate some physiological response to compensate for the damage.

The mechanism of the effect of Leuprolide could be complex. Unfortunately, this study did not indicate whether Leuprolide could affect apical or basal secretion of the 36 kDa protein, or both rates. This newly secreted protein could have any one or more of the following functions: (a) The 36 kDa protein secreted by LC may have an autocrine function to restore normal morphology and function. Conclusive evidence to support this phenomenon would be the demonstration that this protein is in interstitial fluid, in a normal testis (since reduced interstitial space was observed in the Leuprolide treated testes). (b) The 36 kDa protein may be mitogenic as suggested by the presence of many unidentified cells in the interstitial space in the Leuprolide with or without testosterone treated testes. (c) Since it is a well known and

accepted mechanism that communication exists within and between cells in the testis through different secretory products, a paracrine action of the 36 kDa protein cannot be ruled out. Metabolic cooperation between SC and MC has been reported (Hutson, 1983); and the ability of peritubular cells to regulate SC function in coculture has been confirmed (Tung and Fritz, 1980). Thompson et al. (1995) reported that myoid cells influence SC through mechanisms that are different from those that mediate the effects of FSH, and that these influences are due, at least in part, to translational regulation. Gaytan et al. (1994), suggested that macrophages and LC are part of a regulatory loop by which stimulated LC influence macrophages, which in turn modulate LC proliferative activity.

Taking in consideration all these putative functions along with the effects of Leuprolide observed in the morphologic changes of the somatic cells, an hypothesis of the direct effect of this GnRH agonist in the testis can be proposed and a model can be formulated.

LC and not macrophages are favored as a direct target for Leuprolide, since LC possess receptors for LHRH agonists. As a defense mechanism LC synthesized and secreted a 36 kDa protein that may leave the interstitial space and possibly enter the seminiferous tubules via diffusion through spaces between myoid cells junctions. Dym and Fawcett (1970) demonstrated that myoid cell junctions exhibit a 200 A wide interspace and, therefore, are open. Leuprolide may have a secondary effect in somatic cells, MC, and/or SC (outside and inside the ST respectively) through the 36 kDa protein.

The presence of the 36 kDa protein can be questioned, and several observations favor quantitative changes as being the result of alterations in its synthesis and/or secretion and not an artifact. For instance, this protein was shown at different intensities in all sample preparations treated with Leuprolide alone. The testes produce a variety of molecules and many GnRH agonists have marked effects on the function of testicular cells *in vitro* (Verhoeven et al., 1995). In addition, since this protein was found only in the medium of the Leuprolide treated animals, secretion then must have been an active process and not the product of cell lysis which would have cell released of the cytoplasmic proteins. Its appearance in the medium is also unlikely to be a consequence of cell death or cell leakage. If leakage had occurred through SC tight junctions, similar band intensities of each protein (including the 36 kDa protein) would have been seen in both testicular tissue and medium. Dym and Raj (1977) and Hagenas et al. (1978) have shown that SC-SC junctions are remarkably resistant to a variety of deleterious treatments. For example, osmotic changes known to break down the blood brain barrier do not open the junctions between SC (Gilula et al., 1976).

In general, the results of the present study also have shown that the absence or presence of testosterone had no effect on the synthesis and secretion of the proteins, since the same proteins and in similar abundance were observed in the testis tissue in all treated groups and in controls. This is a supported by several findings. Parvinen (1982) has shown that most proteins found in the testis are not under T control, while Niederberger et al.

(1993) and Shubhada et al. (1993) demonstrated that only a few SC proteins are androgen regulated. Sharpe et al. (1992) and Mc Kinnel and Sharpe (1992) have identified several androgen regulated proteins (ARP) and found that ARP-5 protein is negatively regulated by testosterone because its secretion was enhanced in the absence of androgen. Although androgen's mechanism for controlling protein secretion in testes remains obscure, the *in vitro* work of Gorozynska and Handelsman (1995) has provided some clues. These researchers reported that androgen rapidly increased Ca^{2+} levels in isolated SC.

In an effort to obtain additional information about the protein bands found in this study, their apparent molecular weights were compared with those already identified and known to be synthesized by the testes (Table 8). A growth factor with an apparent molecular weight of 14 kDa, named Sertoli Cell Secreted Growth Factor (SCSGF), which is heat and acid stable, is a candidate for the 14 kDa band. This protein was isolated by Niederberger et al. (1993) and found to be secreted *in vitro* by Sertoli cells and this may be a new member of the transforming growth factor (TGF) family. Although MC did not secrete SCSGF, coculture of SC with myoid cells enhanced its secretion two fold demonstrating the importance of intercellular interactions in the maintenance of SC function *in vitro*.

A testicular Interleukin-I (IL-I) which has a molecular weight between 17 and 20 kDa, is a candidate for the 18 kDa protein present in the medium. IL-I is produced by SC but not by interstitial cells (Khan, et al., 1987). The possible

Table 8

Possible identities of TCA precipitated proteins in irradiated rat testicular tissue (t) and medium (m) under different treatments

Protein MW (KDa).	Visualization under different treatments ***	Reference for identification
14	(a,b,c,d) (t/m)	SCSGF (Niederberger et al., 1993)**
16	(a,b,c,d) (m)	
18	(a,b,c,d) (t/m)	Interleukin I (Khan et al., 1987)
24	(a,b,c,d) (t/m)	
25	(a,b,c,d) (t)	Activin (Moore et al., 1994)
28	(a,b,c,d) (t)	
32	(a,b,c,d) (t/m)	Inhibin (Jegou, 1992)
36	(b) (s)	Testin I (Cheng et al., 1989)
43	(a,b,c,d) (t/m)	SPARC (Cheng, 1990)*
49	(a,b,c,d) (t/m)	
55	(a,b,c,d) (t/m)	P-Mos-D (Skinner et al.,1992)
61	(a,b,c,d) (t/m)	
67	(a,b,c,d) (t/m)	Albumin (Melsert et al, 1989) or Testibumin (Cheng et al., 1987)
70	(a,b,c,d) (t)	Prosaposin (Wilson and Griswold, 1979)
77	(a,b,c,d) (t/m)	Testicular transferrin (Onoda and Djakiew, 1990; Skinner and Griswold, 1980)

* Secreted Protein Acidic and Rich in Cysteine
** Sertoli Cell Secreted growth Factor
*** a (control); b (Leuprolide); c (Testosterone Propionate); and d (Leuprolide + Testosterone Propionate).

Note: The individual protein molecular weights listed are the main products of each treated group. Their possible identification is based solely on the similarities of the molecular weights to proteins known to be synthesized and secreted by testicular cells other than germ cells

physiological role of IL-I in the testis remains to be clarified, but it is known that IL-1 suppresses cyclic protein 2 (CP-2), which is usually found in seminiferous testicular fluid and in a lesser amount in SC culture media (Wright and Luzarraga, 1986).

A protein of 25 kDa present in the testes tissue pellet and in the medium may correspond to activin which also has a molecular weight of 25 kDa. Details of activin production by SC has been complicated by the lack of a suitable specific activin assay. However, by western blotting, the presence of a 25 kDa FSH stimulated protein was demonstrated in SC culture (Moore et al., 1994).

The unique 36 kDa protein may correspond to testin I which is also known as CMB-22 and is highly concentrated in the testis. Testin increased when germ cells were damaged, such as by X -ray induced germ cell depletion and busulphan treatment (Jegou et al., 1993). Hence contrary to the positive regulation seen for androgen binding protein (ABP) in tubular fluid, germ cells negatively regulate testin accumulation in testis. Many laboratories have demonstrated that SC synthesize and secret testins *in vitro* (Cheng et al., 1989; Grima et al., 1992).

A 43 kDa band may correspond to a protein known as Secreted Protein that is Acidic and Rich in Cysteine (SPARC). This protein was discovered by western blotting of homogenates of cultured rat SC (Cheng, 1990). SPARC may be involved in sequestering or transporting divalent metal ions in ST (Sage et al., 1989; Vernon and Sage, 1989). The polarity of its secretion by SC

has not been addressed nor have testicular fluid concentrations or the ultimate fate of SPARC been determined. The cyclic appearance of the protein in SC needs to be pursued further to determine if the cytoplasmic accumulation parallels secretion or represent a storage phenomenon prior to a bolus release (Sylvester, 1993).

Another major protein found in the testicular tissue had electrophoretic mobility similar to that of band of 55 kDa is P-Mod-S (Protein that modifies Sertoli cells). Skinner et al. (1988) have isolated P-Mos-S from MC conditioned medium and have found it to be a non-mitogenic paracrine factor involved in interactions between MC and SC. This protein modulates SC functions *in vitro* to a greater extent than any other agent known to influence the cell, but its role *in vivo* remains unclear. Some P-Mos-S may be produced even in the absence of androgen (Verhoeven et al., 1990).

Two possible candidates for the identification of the 67 kDa protein has been proposed: an albumin (Alb)- like protein termed testibumin, which also has a molecular weight of 67 kDa or another albumin found in the rat testicular fluid (Cheng et al., 1987). Testibumin, a modification of albumin (Cheng et al., 1987) is synthesized and secreted by SC and is responsive to FSH and testosterone. It was thought that once testibumin was inside the seminiferous tubule it might replace the function of albumin present in the serum. Albumin has a molecular weight of 67 kDa. Melsert et al (1989) identified it by western blotting of rat Interstitial fluid proteins using an antibody against rat serum

albumin. These researchers concluded that albumin itself could be the main biologically active component of the testicular fluid.

A protein of 70 kDa may resemble prosaposin, which is also a 70 kDa protein. Prosaposin can be found in extracellular fluid. In a pulse-chase analysis, the protein was first present in the SC as a 67 kDa precursor which was subsequently secreted as the 70 kDa mature form and found to be 19% carbohydrate (Wilson and Griswold, 1979). Immunocytochemical studies performed by Sylvester and Morales (1989) have shown that this protein is present in the cytoplasm of SC with no apparent relationship to the stages of the cycle of seminiferous epithelium. The function of prosaposin in the testis remains speculative.

A 77 kDa protein might corresponds to testicular transferrin which has a molecular weight between 75 and 80 kDa (Onoda and Djakiew, 1990; Skinner and Griswold, 1980). Transferrin functions as an iron transport protein, and represents 5% of the total secreted protein in the testis and is a homologue to the serum transport protein. Most SC transferrin is secreted apically from the seminiferous tubule (Morales et al., 1987) and T does not influence the polarity of transferrin secretion.

In summary, the results of the present study have shown that the presence of T did not maintain testicular tissue weight, testicular diameter or Leydig cell morphology. There were changes in the protein profile of the secreted protein profile only in rat testes treated with Leuprolide. Therefore, the changes seen in the morphology and protein profile pattern (especially and

respect to the 36 kDa protein) might be a result of a direct effect in the testicular tissue of a GnRH agonist. The results of the present study could have significant implications in the medical field. For instance, the 36 kDa protein found in this study could become an excellent tool for the development of sensitive methods for the diagnosis of spermatogenesis disruption in normal males. The presence of this protein in the serum might provide important and specific information about Leydig cell and/or ST status and the interstitial space in the testes. In general, it could then provide a means of assessing testicular physiology and pathophysiology in animals *in vivo*. A similar approach could be applied to humans. Similarly, the assessment of male infertility in humans and in animals nearing extinction could be monitored, and better approaches to clinical manipulation and progressive and more effective therapeutic methods developed to treat infertility.

Likewise, since many laboratories are working on the development of contraceptive methods in males, this study may lead to serious reconsideration of the use of GnRH agonists, since GnRH agonist in combination with testosterone is used in many contraceptive trials. The concern addressed in this research is in regard to the observation of a high level of FSH seen in this study following T treatment, with or without Leuprolide, which may have clinical implications in achieving the perfect contraceptive method. In metastatic prostate cancer and other types of cancer the lack of suppression of FSH may

not be a major consideration as long as serum testosterone is absent. Detectable T levels were found in some animals in the Leuprolide-treated group.

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