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Changes in Testicular Morphology in Boars Actively Immunized Against Gonadotropin Hormone-Releasing Hormone

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Alterations in testicular morphology were studied in boars actively immunized against gonadotropin hormone releasing hormone (GnRH). Ten boars were divided equally into two experimental groups (five GnRH-immunized, and five controls). Antibody production was achieved by conjugating GnRH to human serum globulin (hSG). The GnRH-hSG conjugate was emulsified in complete Freund's adjuvant, and administered to boars at 12 weeks of age. Boars were given a booster in incomplete Freund's adjuvant on week 18 and 20. The presence of high antibody titers to GnRH caused luteinizing hormone and testosterone to decline to nondetectable levels. Morphometric examination showed a reduction in percentage volume in Leydig cells/unit testis, seminiferous tubule diameter and seminiferous epithelial height, and an increase in non-Leydig cell interstitial tissue in GnRH-immunized boars compared with controls. Histologic evaluation displayed severe damage of the seminiferous epithelium, absence of spermatids, incomplete cell associations, disruption of Sertoli cells, formation of multinucleated giant cells, and a striking reduction in size and cytoplasmic structures of Leydig cells in GnRH-immunized animals. These results demonstrate the potent inhibitory effects of GnRH immunoneutralization on the boar reproductive system.

Key words: GnRH immunoneutralization, testicular morphology, Sertoli cell, Leydig cell, LH, testosterone, boar.

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It has been well established that gonadotropin hormone-releasing hormone (GnRH) is produced in the hypothalamus and secreted into the portal system (Schally et al, 1973). The anterior pituitary gland recognizes changes in portal blood GnRH concentrations and responds by increasing or decreasing its secretion of gonadotropins (Lincoln, 1979). Depriving the gonadotropes of their main stimulator, GnRH, will prevent gonadotropin release, and in turn inhibit both spermatogenesis and steroidogenesis. A potential means of inhibiting the reproductive system, other than hypophysectomy or lesions of specific nuclei in the hypothalamus, is by intercepting GnRH in the portal blood vessels before it reaches its receptor on the gonadotropes. Immunoneutralization of hypothalamic GnRH by active immunization causes gonadotropin and androgen depletion, and results in impairment of reproductive functions. This has proven to be a valuable tool in the investigation of the reproductive system. Immunoneutralization has been accomplished in a large number of species,

including the rat (Shiota et al, 1981), rabbit (Arimura et al, 1973), marmoset-monkey (Hodges and Hearn, 1977), ram (Schanbacher, 1982), bull (Robertson et al, 1979), rhesus monkey (Chappel et al, 1980), dog (Schanbacher et al, 1983), and boar (Falvo et al, 1986).

In most of the studies done with active production of antibodies against GnRH, endocrine changes as well as morphologic changes (in some species) have been reported. Fraser et al (1974) reported that in rats, the germinal cell population was restricted to Sertoli cells and spermatogonia. However, in the monkey, spermatocytes and spermatids were present but infrequently observed (Chappel et al, 1980). While there is general agreement that testicular morphology is altered, some variation exists among species as to the extent to which this occurs. The purpose of the present study was to extend the investigations of the morphologic changes of the testis following active immunization against GnRH to include the boar. This study reports the changes in testicular morphology that result from depletion of gonadotropin and testosterone (T) in boars actively immunized against GnRH.

Materials and Methods

Design

Ten crossbred boars (Yorkshire × Duroc × Spotted) were divided equally into two groups. One group of boars (GnRH-hSG group) was immunized against GnRH conjugated to human serum globulin (hSG), while the other group was immunized against the carrier molecule (hSG) and served as the adjuvant-carrier control group. GnRH was conjugated to hSG (GnRH-hSG) by carbodiimide reaction (Fraser et al, 1974) and was emulsified by sonication in equal volumes of sterile saline in complete Freund's adjuvant. GnRH-hSG boars were each subcutaneously injected with 200 μg of GnRH-hSG conjugate in complete Freund's adjuvant as described previously (Falvo et al, 1986) 12 weeks after birth. Booster doses in incomplete Freund's adjuvant were given at 16 and 18 weeks of age. Control boars received 200 μg hSG in complete Freund's adjuvant on week 12, and were boosted in incomplete Freund's adjuvant on weeks 16 and 18. Blood samples were obtained for measurements of T, luteinizing hormone (LH) and antibody titers to GnRH by anterior vena cava puncture (MacKenzie, 1961) on week 24. All boars were slaughtered at 24 weeks of age. Each testis was removed, weighed and processed for histologic examination.

Detection of Antibody to GnRH

Antibody titers to GnRH in boars immunized against GnRH were determined on week 12 after primary immunization, as previously described by Schanbacher (1982) and Falvo et al (1986).

Hormone Measurement

Plasma concentrations of LH (Niswender et al, 1970) and T (Falvo and Nalbandov, 1974) were determined in duplicate 200-μl samples by previously described radioimmunoassay procedures. Sensitivities for the respective assays were 10 pg/tube for LH (LER-786-3, which has an LH biologic activity of 0.65 × NIH-LH-Sl U/mg) and 10 pg/tube for T.

Histologic Procedure

One testis from each boar was prepared for light microscopy. Slices of tissue were cut from five different areas of the testis and cut into smaller pieces. Representative histologic sections were prepared from testis fixed in 10% buffered neutral formalin. Sections were dehydrated in alcohol, and embedding was done in JB-4 in beem capsules. Several sections were cut at 2 μm from each testis slice and the sections were mounted individually on slides. A randomly chosen slide from each slice was numbered and stained with Lee's methylene blue basic fuchsin. In addition, sections of testis were processed for electron microscopic examination. Tissue was fixed in 4% glutaraldehyde in phosphate buffer (*p*H 7.2). Post-fixation was accomplished in 1% OsO_4, and embedded in polybed 812 according to Luft's formula (Kushida, 1959; Luft, 1961). Thin sections (60 nm) were cut on an ultra cut E microtome, and sections were viewed on a Hitachi H500H electron microscope.

Morphometric Analysis of the Testis

Blocks from each testis were cut at 2 μm and examined by light microscopy. A minimum of five 2-micron thick sections (all from different blocks) of transversely sectioned testis were selected from each boar, which, on the average, provided an area containing 40 to 45 seminiferous tubules in each section. The counting procedure was designed to provide the information necessary for use in the morphometric equation described by Wiebel and Bolender (1973). Point-counting methods were used to obtain the volume density of Leydig cells, which is the volume of the Leydig cells/unit volume testis. The counting was carried out as follows. Sections were viewed with the 45 × objective of an American Optical microscope. The eyepiece (15 ×) was fitted with a micrometer disc grid (American Scientific Product) containing 11 × 11 squares and 121 line intersection points. The tissue sections and grid were simultaneously viewed. The number of points falling on the Leydig cell (cytoplasm or nucleus) was counted over the whole grid field. The number of "hits"/total number points yielded the volume density. Random fields were counted over the whole specimen, the position of successive fields being determined by steps on the vernier of the microscope stage, without viewing the specimen. As described by Wiebel and Bolender (1973), 12 grid fields/section were counted (7260 test points/testis to find the volume density with a relative error of $< 5\%$). Average volume densities were calculated for each testis and the mean ± SEM for five boars belonging to each group was determined. The volume density of the seminiferous tubule was also measured by point counting with the 10 ×

TABLE 1. Plasma Testosterone, LH, and Antibody Titers to GnRH on Week 24*

Group	N	Testosterone	LH	Antibody titer (1:10,000)
hSG-Adj	5	4.4 ± 1.3†	54.0 ± 4.5†	-
GnRH-hSG	5	ND	ND	76%

*Mean ± SEM; T = ng/ml; LH = pg/ml.
†Significantly different at $P < 0.001$. ND = nondetectable.

objective. The volume density of interstitial tissue was then equal to 1 minus the seminiferous tubule volume density, and non-Leydig cell interstitial tissue was determined by subtracting volume densities of seminiferous tubules and Leydig cells from 1. In addition, the diameter and epithelial height of 15 randomly selected round or nearly round profiles of seminiferous tubules were measured on each slide by optically superimposing a stage micrometer calibrated ocular rectile on the sections. Since the principal interest of the study was to compare the morphology of the normal testis with the testis of GnRH-hSG immunized boars, the possible alteration of volume densities of the measured components due to changes in the volume of tissue blocks during processing and embedding was not taken into account.

Statistics

Hormonal and morphometric data are expressed as the mean ± SEM. Statistical significance was determined by Tukey (Kirk, 1968) at the 95% confidence limit. Data were analyzed by the General Linear model procedure of the Statistical Analysis System (SAS, 1982).

Results

Antibody titer to GnRH rose markedly in response to first and second booster injections in GnRH-immunized boars. On week 24, the percentage of [^{125}I]-GnRH bound to plasma at a final dilution of 1:10,000 was 76% (Table 1). This high antibody titer also coincided with nondetectable plasma concentrations of T and LH (Table 1), indicating neutralization of endogenous GnRH. Upon autopsy, there was a reduction in testicular weight (79%) in boars immunized against GnRH as compared with controls (Table 2).

Light Microscopic Appearance

Histologic evaluation of the testis showed a remarkable difference between the control and GnRH-immunized boars. In control boars, the interstitial tissue was completely filled with distinct Leydig cells, and seminiferous tubules showing various stages of the cycle of the seminiferous epithelium were present (Fig. 1). In contrast, as indicated by histologic observation and plasma T levels, spermatogenesis was impaired in the GnRH-immunized boar testis. While some areas of the seminiferous tubules displayed little damage, other areas depicted disruption of the epithelium, including Sertoli cells, incomplete cell associations, and complete absence of post-meiotic germ cells and spermatids (Fig. 2). Although some pachytene spermatocytes were present in the seminiferous tubules of GnRH-immunized boars, they seemed to be degenerating. These cells appeared small and dense with evidence of shrinkage.

Leydig cells in control boars appeared large with round or spherical nuclei, prominent nucleoli, and cytoplasmic inclusions (Fig. 3). However, these cells appeared disrupted in immunized boars (Fig. 4), and were smaller in size than in the controls. Their shapes were often irregular, the plasma membranes were not well defined, and much less cytoplasm was visible surrounding the nuclei. Small, dense and pyknotic nuclei were also present, indicating degenerative changes.

Electron Microscopic Appearance

Electron microscopic appearance of the control boar testis is shown in Fig. 5, while that of the GnRH-immunized boar testis displaying intermediate and severe damage of the seminiferous tubule is shown in Figs. 6 and 7, respectively. Some parts of the testis of GnRH-immunized boars showed intermediate

TABLE 2. Morphometric Data of Testicular Components in Control and GnRH-immunized Boars*

Group	N	Testicular Weight (g)	% Volume of Leydig-Cells/ unit Testis	% Volume of Seminiferous Tubules/unit Testis	% Volume of Non-Leydig Cell Interstitial Tissue/unit Testis	Seminiferous Tubule Diameter (μm)	Seminiferous Tubule Epithelial height (μm)
hSG-Adj	5	578.0 ± 41.8^{A}	23.6 ± 2.9%^{A}	64.2 ± 2.9%^{A}	12.2 ± 3.1%^{B}	252.0 ± 10.1^{A}	64.1 ± 5.2^{A}
GnRH-hSG	5	123.2 ± 23.5^{B}	5.5 ± 0.7%^{B}	60.7 ± 2.8%^{A}	33.7 ± 2.8%^{A}	177.0 ± 11.6^{B}	40.5 ± 4.5^{B}

*Mean ± SEM.
^{A,B}Values without common superscripts differ ($P < 0.001$) between groups (vertical comparison).

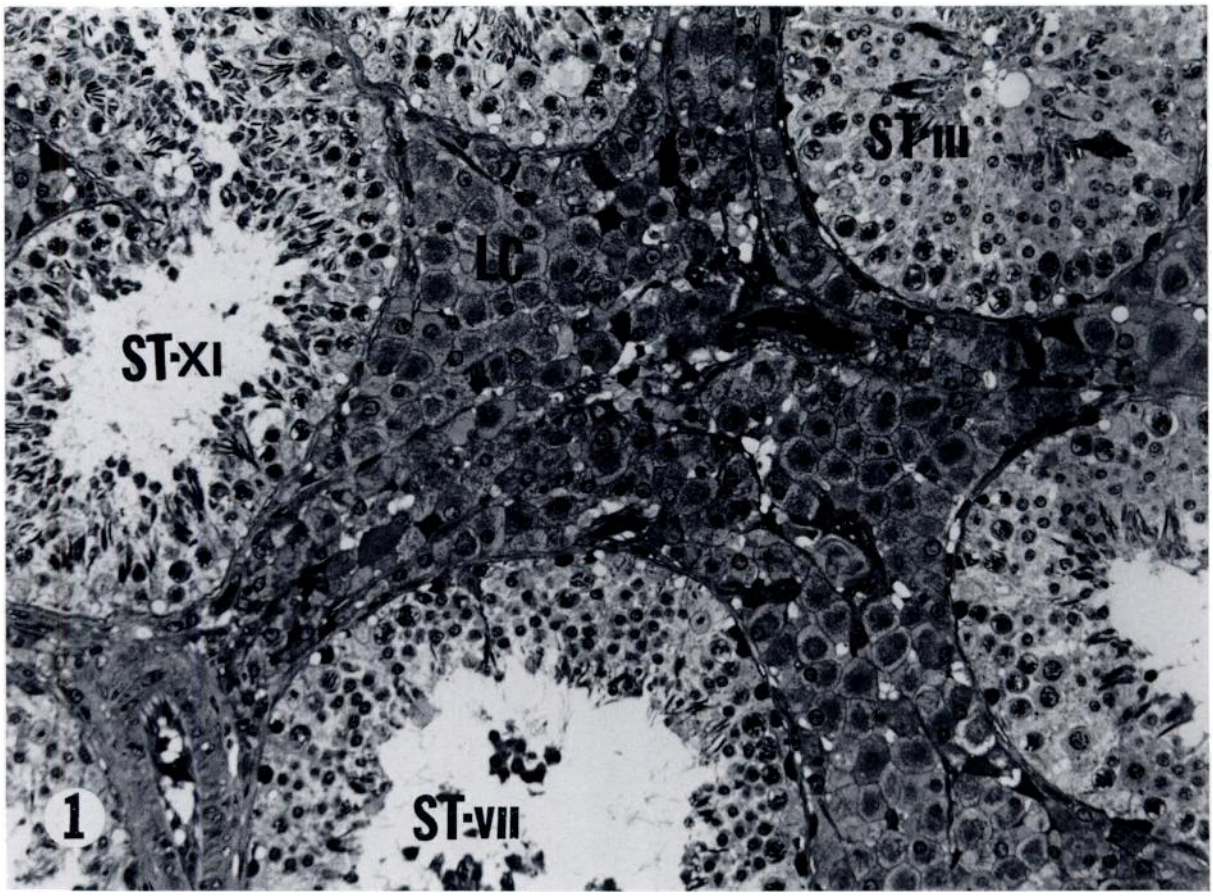


Fig. 1. Light micrograph of testis from a control boar showing seminiferous tubules (ST) with spermatids present. Leydig cells (LC) are very distinct and generally arranged in clusters between seminiferous tubules. ST-III, VII, and XI are stages of spermatogenesis in the seminiferous tubules. Section was stained with Lee's methylene blue basic fuchsin. (× 200).

damage of the seminiferous tubule, such as vacuolization of Sertoli cells, increased extracellular space, and multinucleated spermatids (Figs. 6 and 8). In other sections, the seminiferous tubules displayed severe damage (Fig. 7), including a large lumen and watery Sertoli cell cytoplasm, with no spermatozoa or post-meiotic cells.

Quantitative Analysis

Morphometric analysis showed a 30% reduction in seminiferous tubule diameter in GnRH-immunized boars compared with controls (Table 2). The seminiferous tubules appeared to be reduced in size in these animals, but the volume density did not differ from control boars. However, when the relative mass was estimated (by multiplying the weight of the testis by volume density; Ewing et al, 1979), it represented a 79% decrease in seminiferous tubules compared with the controls (Table 2).

The volume density of Leydig cells was significantly reduced (79%) in immunized animals (Table 2). In terms of estimated mass, there was a 95% reduction of Leydig cells in immunized boars compared with controls. The volume density of the interstitial tissue was not significantly different in both groups. However, when the volume density of non-Leydig cell interstitial tissue was calculated, there was a 276% increase (Table 2) in immunized animals compared with controls.

Discussion

The role of T in the qualitative and quantitative maintenance of both spermatogenesis and testicular morphologic integrity has been established (Desjardins et al, 1973). Using an indirect approach to study the hypothalamic-pituitary-testicular axis by active production of antibodies against GnRH, reports from different species, such as the dog (English et al,

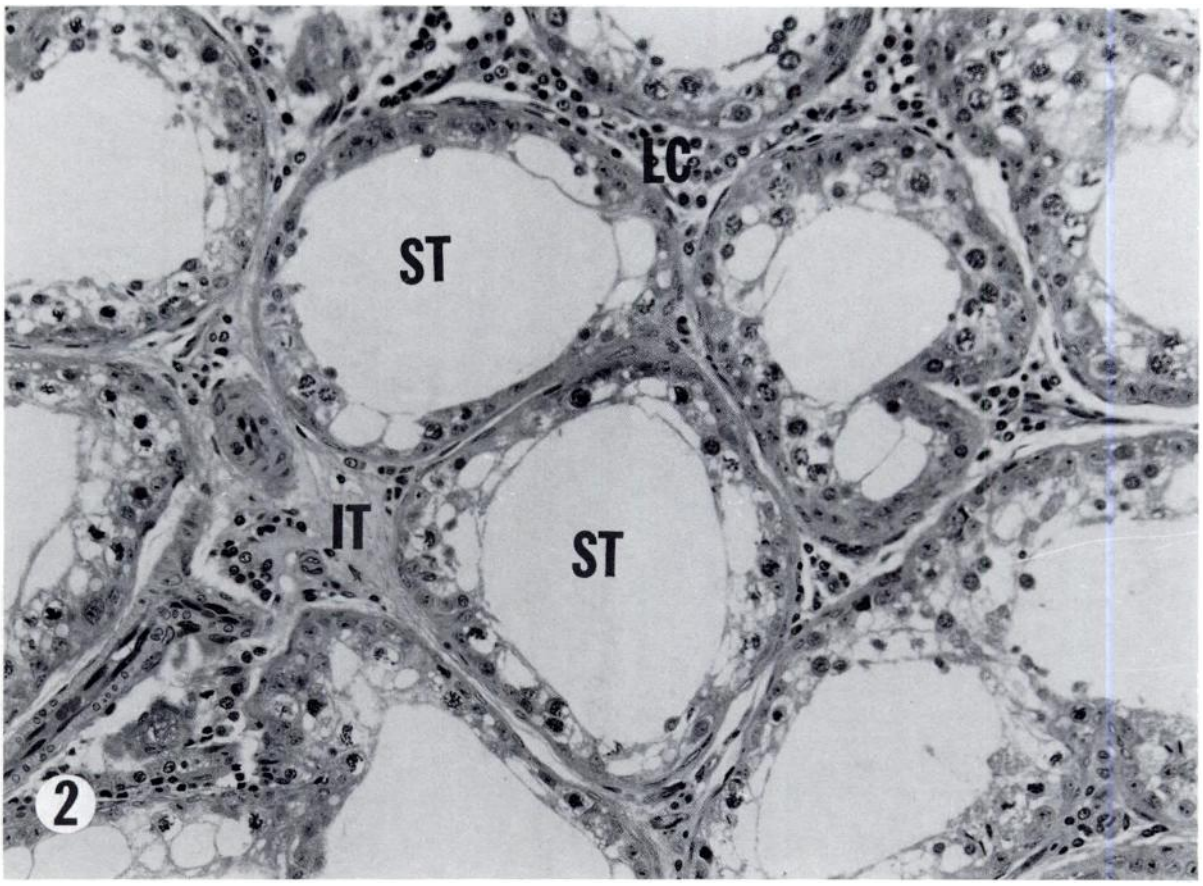


Fig. 2. Light micrograph of testis from GnRH-immunized boar. Note the disruption of seminiferous epithelium, complete absence of spermatids, reduction in size of the seminiferous tubules (ST) and Leydig cells (LC) and an increase in interstitial tissue (IT) compared with controls (Fig. 1). Section was stained with Lee's methylene blue basic fuchsin. (× 200).

1983) rhesus monkey (Chappel et al, 1980) rat (Fraser et al, 1974) and ram (Schanbacher, 1982) have described morphologic alterations in the testis; however, these morphologic changes have not been reported in boars.

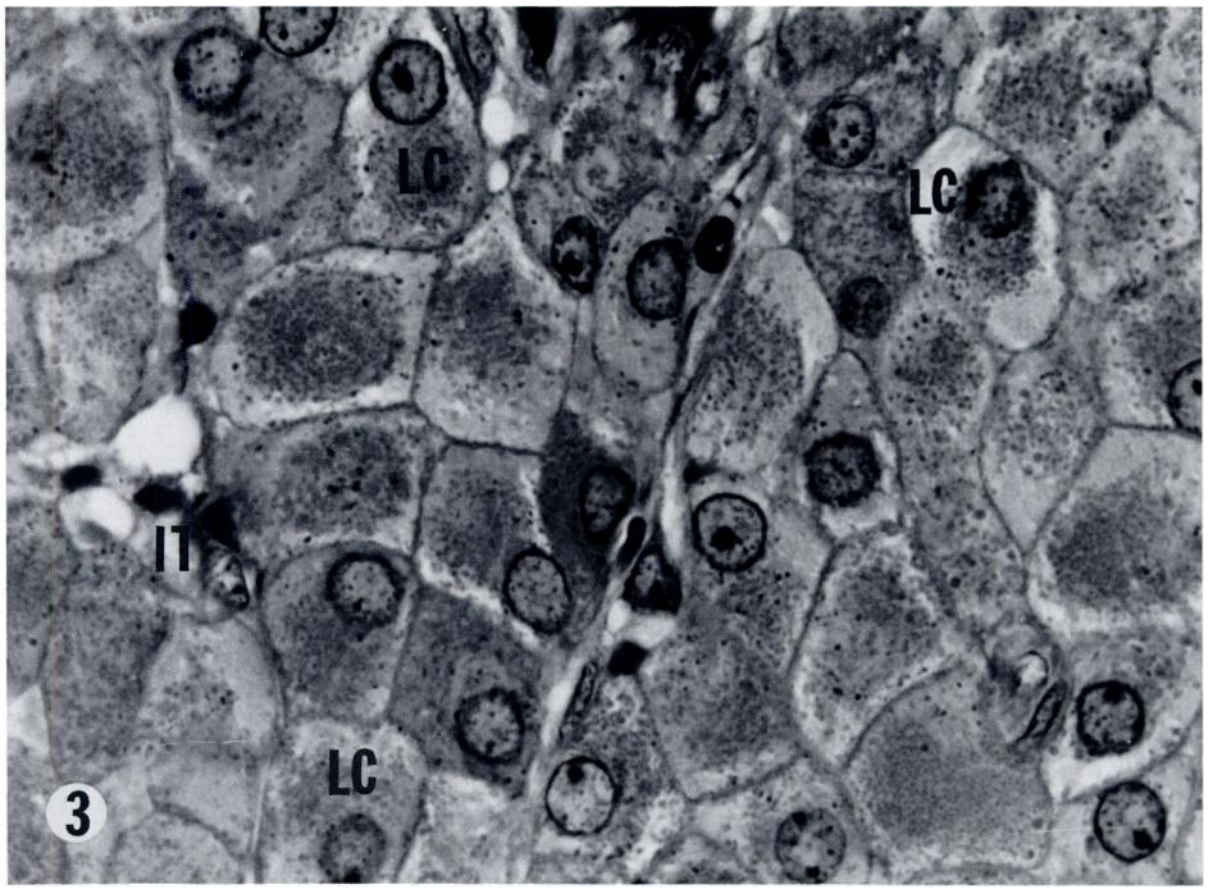
Active immunization against GnRH in this study clearly demonstrates that boars are capable of generating high GnRH antibody titers. When chronically sustained, these antibody titers can result in depletion of LH and T, with concomitant changes in testicular structure and function. Having succeeded in reducing plasma T levels to those observed following hypophysectomy, there was a striking reduction in testicular weight at autopsy, and histologic alterations of both the seminiferous epithelium and Leydig cells.

The observed reduction in plasma LH and T levels, and of testicular weights in GnRH-immunized boars correlated very well with the morphologic appearance of the Leydig cells and seminiferous tubules. Since the rate of T synthesis and secretion by Leydig cells is primarily dependent upon LH secretion, depletion of LH as a result of GnRH immunization reduced the size, number, and activity of these cells, and resulted in T depletion.

Selective withdrawal of follicle stimulating hor-

Fig. 3. Light micrograph from control boar testis. Leydig cells (LC) are arranged in clusters with well defined plasma membranes, round nuclei and cytoplasmic inclusions. (IT = interstitial tissue). Section was stained with Lee's methylene blue basic fuchsin. (× 1000).

Fig. 4. Light micrograph from GnRH-immunized boar testis. Pyknosis of Leydig cells (LC) with less visible cytoplasm, dense nuclei, and reduction in size are seen when compared with control (Fig. 7). (PN = Pyknotic nucleus; IT = interstitial tissue). Section was stained with Lee's methylene blue basic fuchsin. (× 1000).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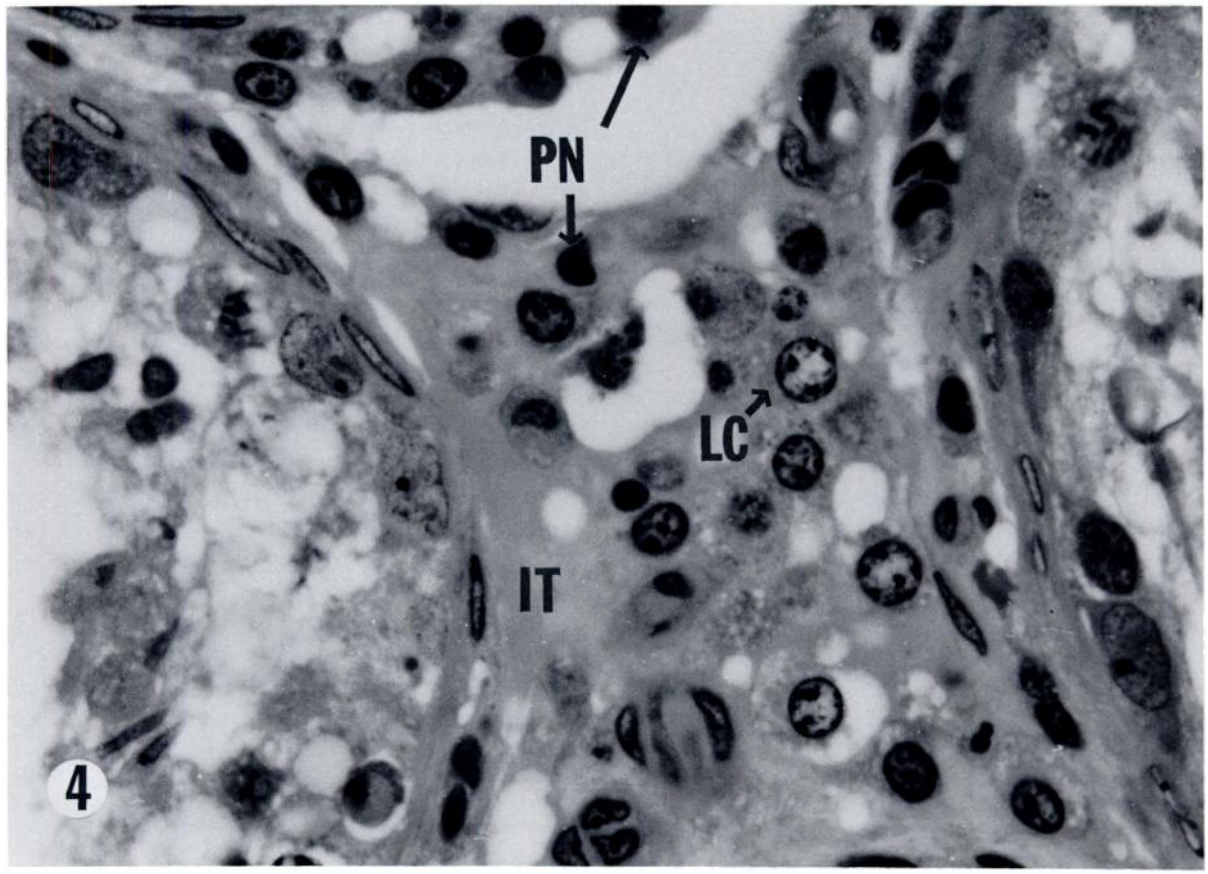
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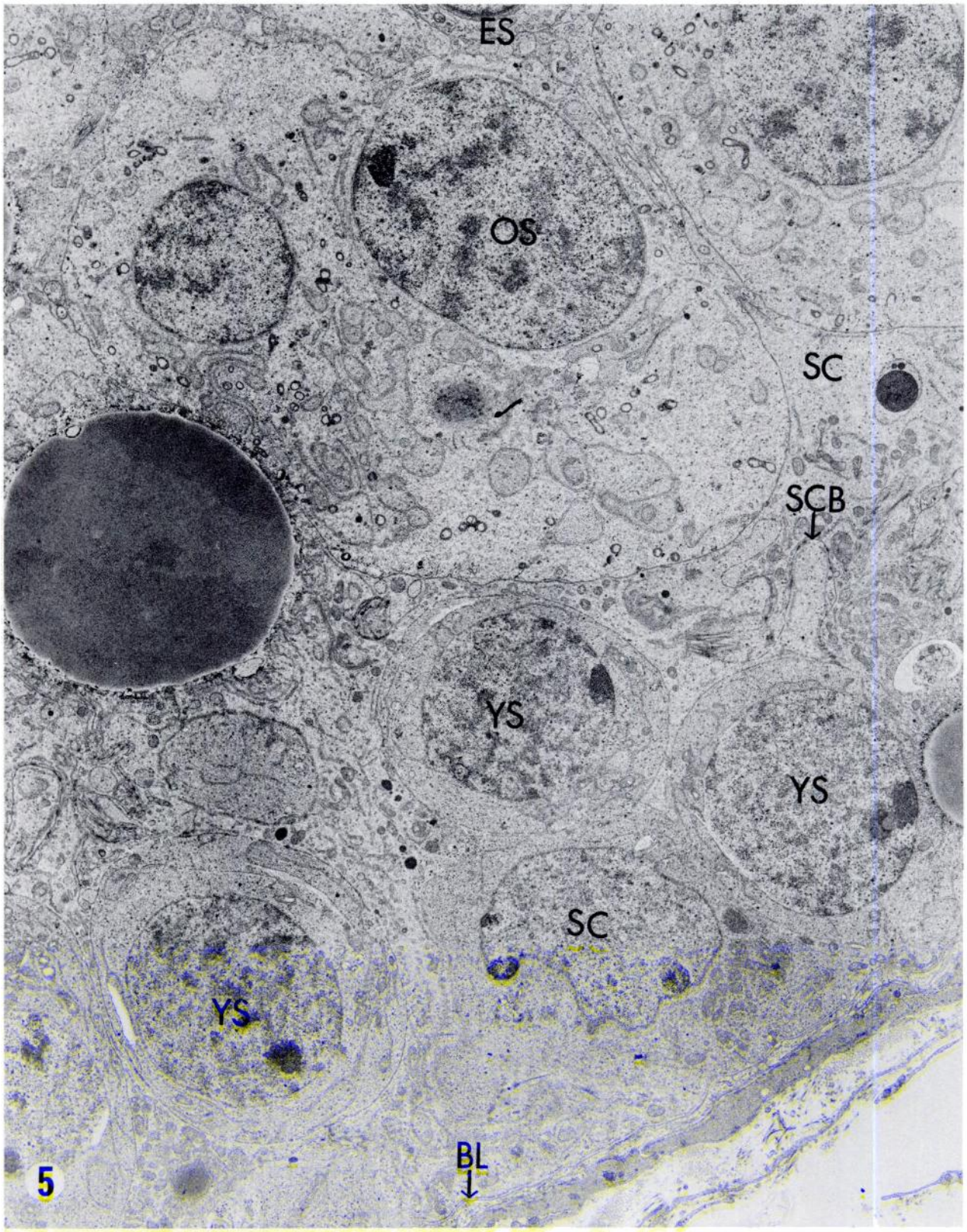

Fig. 5. Electron micrograph of testis from control boar showing seminiferous epithelium. Note Sertoli cell (SC), young spermatocytes (YS), and Sertoli cell barrier (SCB) separating the young spermatocytes from old spermatocytes (OS) and early spermatids (ES). BL ≡ basal lamina. (× 4860).

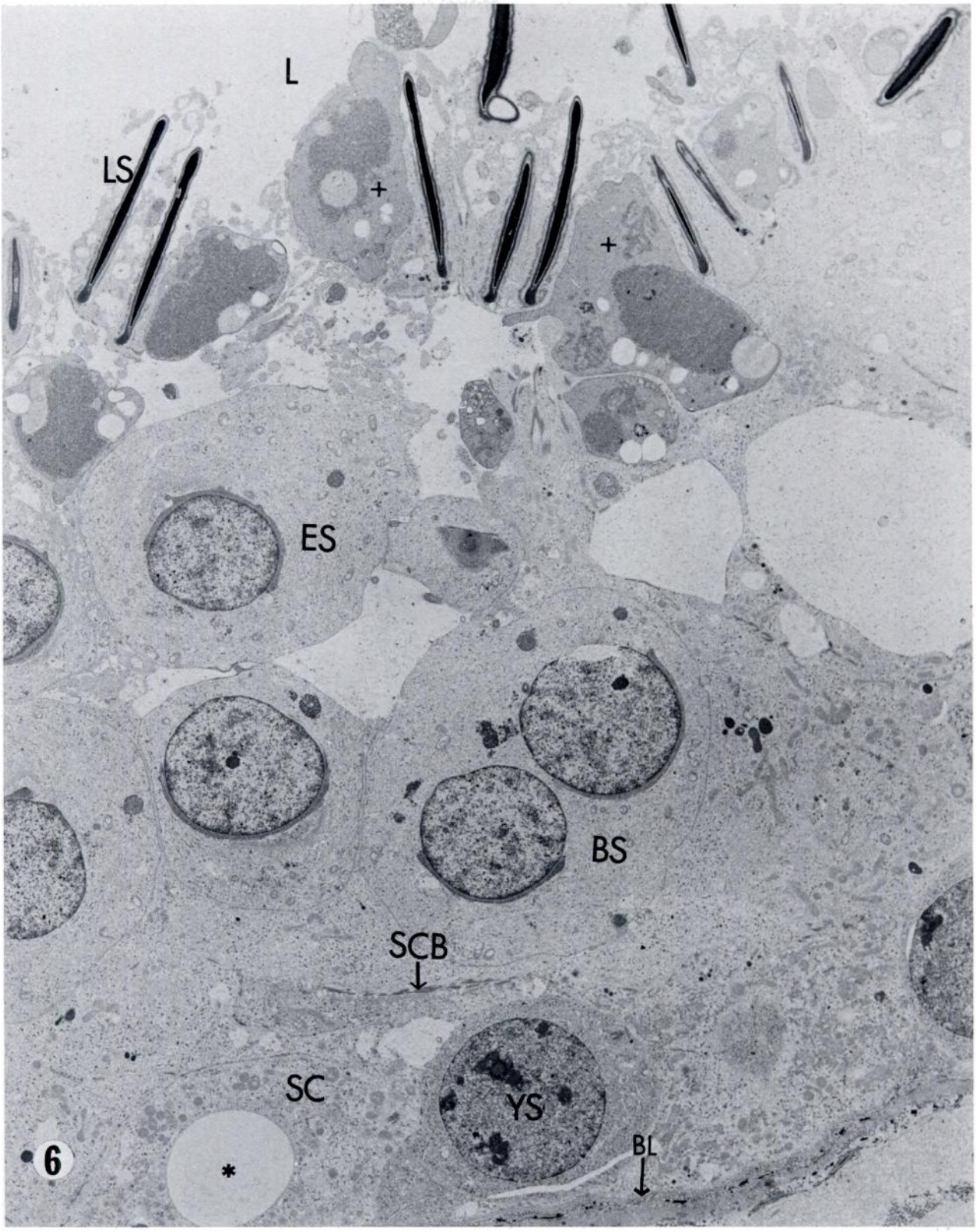

Fig. 6. Electron micrograph depicting intermediate damage of the seminiferous epithelium in GnRH-immunized boar. Sertoli cell (SC) contained vacuoles (*). Also note the cytoplasmic lobes (+) and the formation of binucleated spermatid (BS). Other structures include basal lamina (BL), Sertoli cell barrier (SCB), early spermatids (ES), late spermatids (LS) and lumen (L). (× 4860).

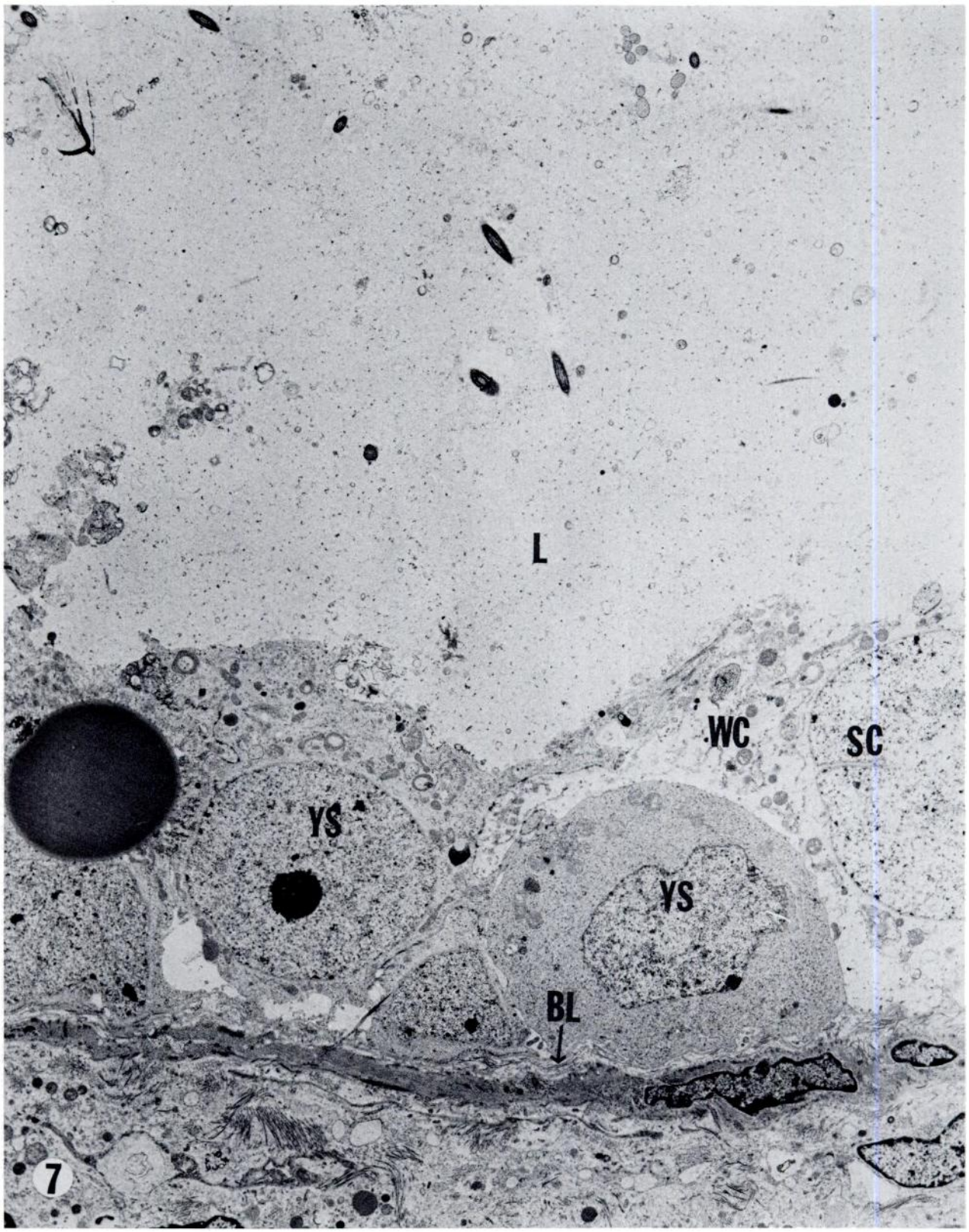

Fig. 7. Electron micrograph of testis from a GnRH-immunized boar depicting severe damage of the seminiferous epithelium. Note the complete absence of postmeiotic cells, and the increase in the size of the lumen (L). (BL = basal lamina; YS = young spermatocyte; WC = watery cytoplasm of Sertoli cell; SC = Sertoli cell). (× 4860).

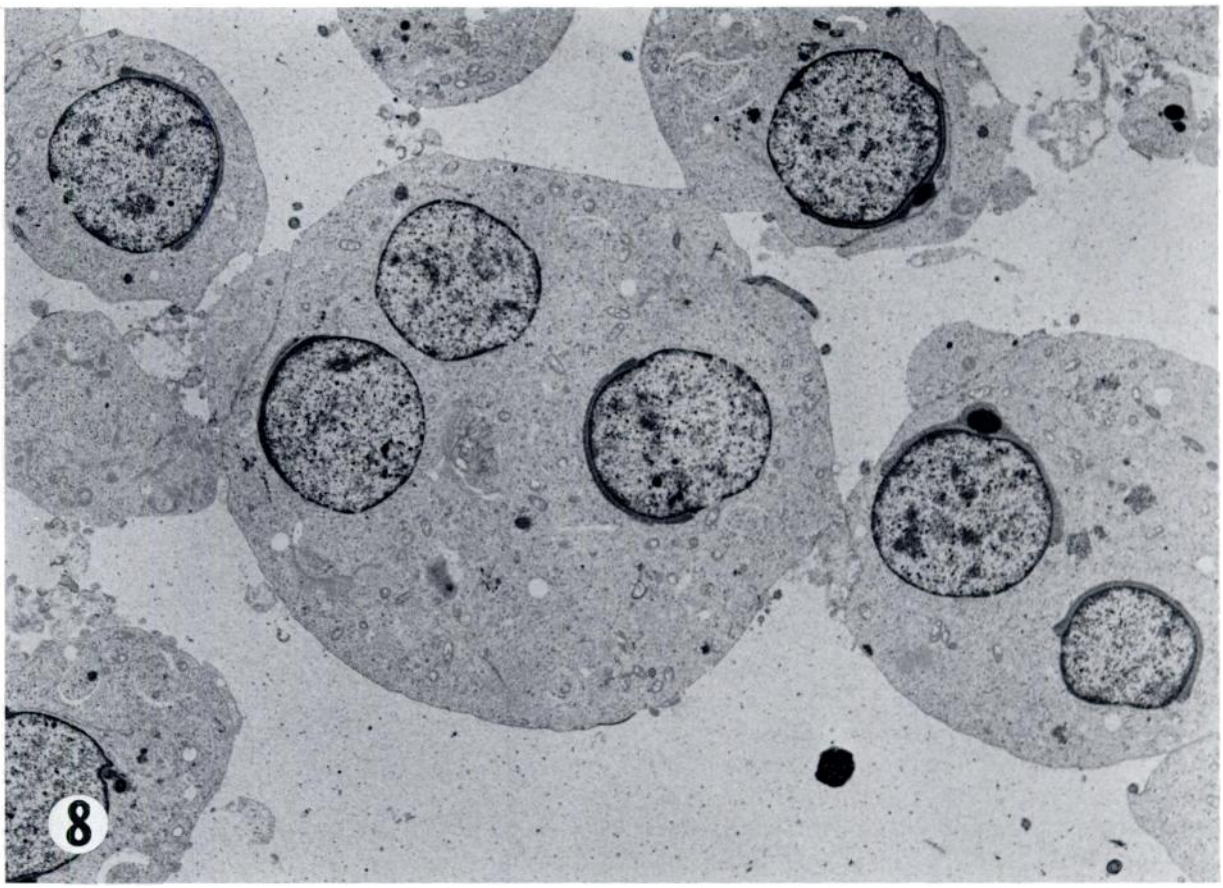

Fig. 8. Electron micrograph of multinucleated spermatids from GnRH-immunized boar testis. (× 4050).

mone (FSH) was found to be deleterious to normal spermatogenesis in intact immature rats (Madhwa Raj and Dym, 1976). Therefore, FSH may also have contributed to the changes seen in the seminiferous epithelium in this study. It is uncertain whether the effects of active immunization against GnRH included inhibition of FSH secretion or only impaired that of LH. The reduction in seminiferous tubule diameter and epithelial height, absence of spermatids and postmeiotic cells, vacuolization and disruption of Sertoli cells in GnRH-immunized boars all support the curtailment of spermatogenesis.

The numbers of germ cells were reduced following withdrawal of T when compared with controls. However, while some of the germinal cells present in GnRH-immunized boars appeared normal, some acquired bizarre shapes, and some possessed multinucleated spermatids. Multinucleated giant cells have been noted in the rat seminiferous epithelium following treatment with several chemical agents (Fang and Anderson, 1976; Chapin et al, 1983; 1984), as well as in vitamin A deficiency (Sobhon et al, 1979). The origin of these cells is still unknown; however, they may have arisen as a result of karyokinesis without cytokinesis (Reddy and Svoboda, 1967; Mitranond et al, 1979), or the result of fused germ cells (Pryor and Ferguson, 1950). In a more recent study where multinucleated spermatids were very prominent in the aging human testis (Nistal et al, 1986), it was suggested that multinucleated spermatid formation was due to cell fusion and represented a degenerative process. Since there is a decrease in androgen levels during old age in men (> 70 years of age), perhaps androgen depletion may be related to cell fusion and the formation of multinucleated spermatids as observed in our study.

Normal Sertoli cells were observed in rabbits after immunization against GnRH (Arimura et al, 1973). In contrast to this finding, Sertoli cells seem to be more affected in our study, suggesting that these cells are principal targets for the action of T in the testis. This also indicates that Sertoli cells in boars are

more sensitive to testosterone deprivation. Vacuolization, watery cytoplasm, and decreased Sertoli cell height were prominent. Similar observations of decreased Sertoli cell height has also been reported following selective withdrawal of LH (and T) in rats immunized against anti-LH (Madhwa Raj and Dym, 1976). These morphologic changes resulting from T depletion, together with the fact that Sertoli cells provide the microenvironment in which germ cells develop, support the concept that the damaging actions of many agents upon the seminiferous epithelium may first affect the Sertoli cells before the germ cells, and also support the absence of postmeiotic cells and spermatids that we observed.

The tight junctions between adjacent Sertoli cells are known to allow the osmotic gradient necessary for the passage of fluid from the interstitial capillaries to the tubule lumen (Setchell, 1970). The presence of luminal spaces in seminiferous tubules is an established function of the Sertoli cells (Madhwa and Dym, 1976). Chronic hypophysectomy will result in disappearance of the lumen, indicating decreased fluid production by Sertoli cells. This phenomenon was noted when anti-LH was given to immature rats (Madhwa and Dym, 1976). In contrast to this finding, the seminiferous tubules in GnRH-immunized boars in our study possessed large lumina, suggesting that although the Sertoli cells seemed disrupted, their tight junctions may still have been intact. However, maintenance of high antibody titers to GnRH for a longer period of time may create the situation observed after long-term hypophysectomy (ie, cessation of fluid production by the Sertoli cells).

In view of the various sequelae to active immunization against GnRH on testicular morphology, we conclude that LH depletion affects the structure and function of Leydig cells, and the corresponding depletion of T (as a result of LH depletion) and possibly FSH exerts deleterious effects on Sertoli cell architecture and germinal cells, and causes a curtailment of spermatogenesis.

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WHO Reference Material (+) and (−) Gossypol

Chemists collaborating with WHO have made it possible to distribute a limited number of ampoules containing 20 mg of the (+) and (−) enantiomers of gossypol of high chemical and optical purity for studies in chemistry and on the mechanism of action of (−) gossypol on testicular cells *in vitro*. Limited quantities (1 gm per ampoule) are also available for animal studies on the mechanism of action of (−) gossypol in the male on receipt of a brief protocol outlining the experiments to be undertaken. Novel features should be emphasized. Note that this material is NOT for human use. Please address requests to: The Task Force Manager, Methods for the Regulation of Male Fertility, Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization, 1211 Geneva 27, Switzerland.

The WHO Special Programme of Research, Development and Research Training in Human Reproduction should be acknowledged in any publication arising from the use of this material.