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Computerized microscopy studies concerning the structural biology of the rooster's testes during its reproduction season

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Abstract

The use of reproduction biotechnologies requires the understanding of the functional structures of the rooster's testicular parenchyma during its reproductive season by obtaining new data about the evolution seminal line cells, of the Sertoli cells and of the cells of the interstitial Leydig glands. Electronmicroscopy researches point out studies of the cellular organelles and it is called the identification of the spermatogenesis steps (the complete spermatogenesis process until the second meiotic division during which the spermatids are formed). It is traced the study of the Sertoli cell and the type of junctional complexes it realizes with the basal membrane of the seminiferous tube. This study has the relevance for the correct evaluation of their productive levels following the usage during reproduction of the most valuable specimens. This data gives a scientific contribution to the veterinary andrology, the understanding of the spermatogenesis developing process and of the productive yield of this process with the purpose of using the reproduction biotechnologies.

Keywords

Testicular parenchyma, seminiferous tubules, Sertoli cells, spermatogonia, spermatocytes, spermatozoa.

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Introduction

Testis is a compound tubular gland with an exocrine and an endocrine functions. The exocrine function is the production of spermatozoa by the lining epithelium of the seminiferous tubules. The endocrine function is the production of the male sex hormone, testosterone, by the specific interstitial, Leydig cell in the intertubular connective tissue (Aughhey E. & Frye F. 2011 [3]). The structural biology of the testicular parenchyma in the roosters constitutes the morphological and physiological base of the sperm production in this species, so that the understanding of the cells that are part of the seminiferous tubes allows the modelation of the optimum conduct concerning the feeding, maintaining and exploring with the goal of obtaining a seminal material the will pass all the quality requirements. The tunica albuginea is a solid capsule of dense irregular connective tissue. It consists predominantly of collagen fibers, a few elastic fibers, and myofibro-blasts that meander along the branches of the testicular artery; a network of anastomosing veins constitutes the vascular layer of the tunica albuginea (Razi M, 2010 [12]). Seminiferous tubules are lined with stratified spermatogenic epithelium (germinal epithelium), surrounded by the lamina propria and connected at both ends to straight testicular tubules by a specialized terminal segment (Bergmann, 2006 [6]). Epithelium of the convoluted seminiferous tubule includes supporting cells and spermatogenic cells. The supporting cells (Sertoli cells), located in the basal region of the epithelium, surround the adjacent spermatogenic cells. Tightly bound to other supporting cells, they create a blood-testis barrier which shields the developing spermatogenic cells from blood-borne influence. Spermatogenic cells in various stages of development comprise the balance of the cell population (Cornilă N. (2000-2001 [7]). The basally located spermatogonium is capable of mitosis. The largest cell, the primary spermatocyte, undergoes the first meiotic division while the secondary spermatocyte completes the second meiotic division. The resulting spermatid is haploid and becomes the spermatozoon which represents the final developmental stage.

These result were in agreement with Bacha W. and Bacha L., how described that the spermatogenesis in birds, as in mammals, involves a series of divisions of spermatogonia, resulting in primary spermatocytes and secondary spermatocytes, both of which undergo meiotic divisions, culminating in the evolution of spermatids (Bacha W.J. & Bacha L.M. 2000 [4]). Along these cells, sustentacular (Sertoli) cells, also observed. On average, 12 layers of cells were seen in the epithelium of seminiferous tubules. The spermatogonal cell layer showed some variation between different birds and these cells were arranged in between one and three layers' however, the spermatocytes (A and B), secondary spermatocytes and spermatids were arranged in 2, 3-4 and

3-5 layers respectively. The interstitium of the testes was made up of loose connective tissue, which contained Leydig cells in close vicinity to the arterioles and capillaries. The mode of distribution of these cells was not uniform. A relatively high population of mononuclear immune cells, fibroblasts and fibrocytes were observed in close proximity to the blood vessels (Razi, M et. al., 2010 [12]). Testosterone is produced and secreted by the Leydig cells. The hormone regulates the male reproductive system by the negative feedback mechanism of the hypothalamus-pituitary-testes axis. We found that the decrease of testosterone plasma level of the aging, low fertile roosters is characterized by Leydig cells showing reduced number and volume of the mitochondria, were testosterone biosynthesis initiates by cholesterol cleavage, rough endoplasmic reticulum involved in protein synthesis and smooth endoplasmic reticulum involved in testosterone secretion.

Moreover, spermatogenesis remains normal and the spermatogeneous cells show regular ultra-structure (Rosenstrauch Avi, 2007 [13]).

The Leydig cells were found single or in small clusters, primarily in the larger interstitial spaces, they are recognized by their small, round nucleus and an acidophilic, often foamy cytoplasm. Aire (2002 [1]) suggested the interstitium is relatively compact in birds except in the ostrich in which it forms a loose and 'oedematous' connective tissue. Seminiferous tubules of birds are dissimilar to those of mammals by forming highly and complexly anastomotic, non-blind-ending network of tubules and this probably responsible for the lack of connective tissue septa, as well as the non lobulation of the avian testis.

The research done on the testicular parenchyma in the rooster during its reproductive season have highlighted a seminal epithelium sustained by a thick basal membrane formed by a lamina lucida, electronodense, with a thickness of 10 nanometers, with an omogenous aspect and crossed by a rare, thin filaments. Lamina densa has a thickness of 20-30 nanometers and it is formed by abundant thin filaments included in an amorphous matrix. Lamina reticulata does the pass to the conjunctive tissue.

Materials and Methods

The researches were conducted on testes harvested from cock, race-variety white Leghorn, în perioada reproductivă normally developed, clinically healthy, vaccinated, macroscopically and microscopically examined. It made an experiment which included two lots of cocks, each lot consisting of 4 copies each, from each individual were collected pieces of testicular parenchyma from skull pole, caudal, medial edge and lateral edge. The inspection of histological preparations were made on permanent histology, processed by usual histological techniques and colored by hematoxylin-eozine method, Giemsa slow sections, Tanzer-Unna and P.A.S histochemical techniques.

The experimental studies were performed on 12 testes of the rosters of different ages and breeds using transmission electron microscope (Transmission Electron Microscopy-TEM).

Electronic equipment and investigative techniques have created working conditions for obtaining optimal information to be completed painting ultrastructural morphology of the seminal epithelium cells that make up the swine species. Tissue fragments were fashioned pieces with dimensions of 0.25 / 0.25 / 0.5 mm, were fixed in phosphate buffered saline (PBS) with 2.5 glutaraldehyde. The fixing after washing was performed with osmium tetroxide (OsO₄), dehydration, propylene oxide and then clarify the inclusion of pieces in the mixture of 812 + DDSA Epona (7 ml) + DMP30 (0.15 ml). Inclusion was made in natural rubber moldes or in special capsules. After an hour of the shift was made under lupan inclusion tissues. Division of ultra fine preparations resulted in obtaining sections with a thickness of 300-500 Å were then deposited ultrafine sections where intake grille of their surface which are covered with double membranes (formwar and carbon) by touching the grid sections. It performs a dual contrasted with Reynolds solution and then examining the sample transmission electron microscope at 60-75 kV acceleration and a magnification of 10,000 and 100,000 times.

Results and Discussion

During puberty, differentiation of cell sustentacular is accompanied by a morphological transformation and loss of mitotic ability (at adult Sertoli cells no longer divide). Inside the testicular parenchyma appear muscular type arteries and in seminal epithelium predominate are spermatocytes by first order. Appear spermatocytes by second order and spermatozoides. Secondary spermatocytes are located more centrally from primary spermatocytes, were short-lived, intermediate size and spherical nucleous.

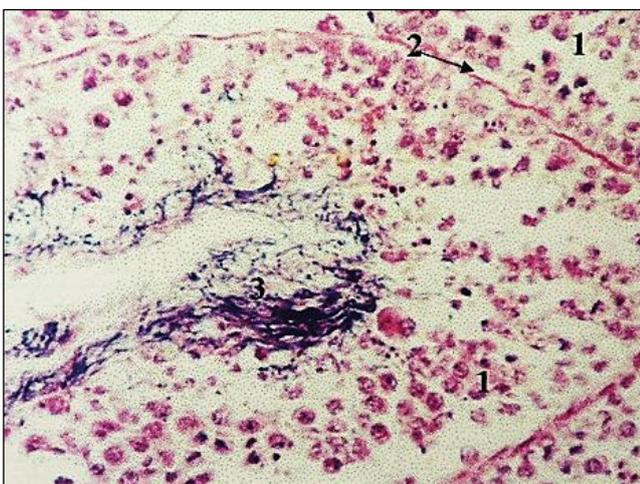


Figure 1. Seminiferous tube at cocks by 120 days, P.A.S., ob 40x.

1- seminal epithelium; 2- basement membrane;
3- spermatozoa in the lumen.

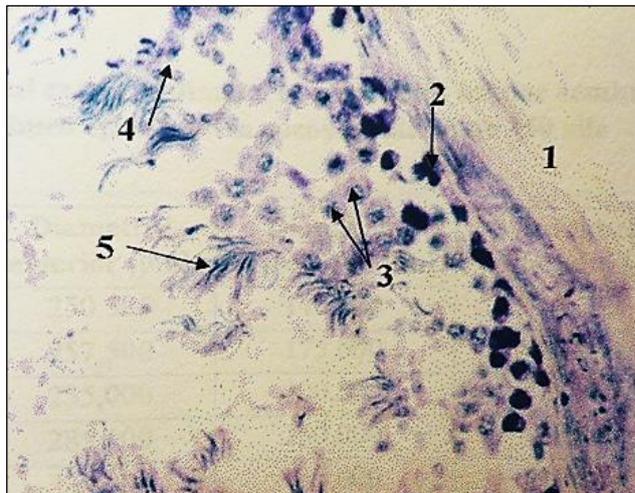


Figure 2. Testicular parenchyma at cocks by 120 days, Giemsa, ob 40x.

1- albuginea; 2- spermatogonia; 3- primary spermatocyte;
4- spermatid; 5- tails of sperm.

By mitotic division pass in spermatid, round cells with spherical nucleous, possessing a haploid number of chromosomes and resembling with secondary spermatocytes, but smaller. At the age of 120 days has seen an increase in both the outer diameter of seminiferous tubules, lumen diameter, and a significant increase in seminal epithelium height (Figure 1).

The formation of the hemo-testicular barrier was correlated with the appearance of the haploid germ cells. The complete subdivision of the seminiferous tubes leaving only the spermatogonia inside the opened compartment, was realised within the tubes that were containing the elongated spermatids during their maturation phase. During the final phases of the spermatids maturation into spermatozoa, the residual cytoplasm and the organelles are removed by a body of cytoplasm attached to the caudal portion of the developing spermatid (Figure 2). These cytoplasmic debris are called residual bodies and they are phagocytosed by the Sertoli cells and are degraded by the cell's lysosomes.

At the age of 120 days, in the seminal epithelium can be observed all the cells in the seminal line: spermatogonia, spermatocytes, spermatid and spermatozoa. A PAS-positive reaction is observed in the basal membrane of the seminiferous tubes and the reticular fibers surrounding the blood vessels. In the sections stained with Tanzer-Unna there show up the elastic fibers in the basal membrane of the seminiferous tubes. On top of the basal membrane we find the stratified seminal epithelium that has a characteristic columnar display, on top of this columns standing the late spermatid and the spermatozoa.

The spermatocytogenesis is the phase in which the diploid spermatogonia are situated in the most peripheral position of the seminiferous tube. The stem spermatogonia have an elliptic shape with an eccentric oval and pale nucleus, that begin to divide mitotically resulting in a Type A spermatogonia, a intermediary spermatogonia (I) and a Type B spermatogonia. The Type A spermatogonia has an

oval nucleus, with proeminent nucleoli that tend to become peripheral. The Type B spermatogonia is a spherical cell, with a spherical nucleus and less proeminent nucleoli and the contact zone with the lamina basalis is more limited. From the mitotic division of a Type B spermatogonia the primary spermatocytes are formed. These are the largest cells within the seminal line, localized in an intermediary position, between the spermatogonia and the spermatid, their nuclei are big and round with big nucleoli. They lose step by step the contact with the lamina basalis and move towards the adluminal compartment through the intercellular junctions between the Sertoli cells. The secondary spermatocytes are located apically, above

the primary spermatocytes, they have a short life span, intermediary dimensions and spherical nuclei. Through mitotic division they transform into spermatid, round cells with spherical nuclei that have a haploid number of chromosomes and are very much alike with the secondary spermatocytes, but have smaller dimensions. At the age of 120 days, the outer diameter of seminiferous tubules has reached an average of 217, 575 μm , lumen diameter of seminiferous tubules averaged 104, 716 μm , and the height of epithelium is double, noting average of 56, 429 μm (Table 1).

Outer diameter of seminiferous tubules, lumen diameter and height of epithelium at cock by 120 days old:

Table 1

SEMINIFERIOUS TUBULES	OUTSIDE DIAMETER (μm)	LUMEN DIAMETERS (μm)	EPITHELIUM HEIGHT (μm)
1	218,182	83,838	67,172
2	215,151	78,788	68,181
3	168, 687	95,960	36,363
4	169,697	71,717	48,990
5	191,913	95,960	47,976
6	187,879	100,000	43,939
7	277,778	145,454	66,162
8	318,182	148,485	84,848
9	157576	80,500	38,538
10	270,707	146,465	62,121
MEDIA	217,575	104,716	56,429

On the 180 days old roosters morphometric determinations were done to evaluate the outer diametere, the diameter of the lumen inside the seminiferous tubes and the height of the epithelium. The outer diameter and the luminal diameter inside the seminiferous tubes has grown

much comparing to the age of 180 days and the height of the epithelium has reduced as it is observed in the following table.

Outer diameter of seminiferous tubules, lumen diameter and height of epithelium at cock by 180 days old:

Table 2

SEMINIFERIOUS TUBULES	OUTSIDE DIAMETER (μm)	LUMEN DIAMETERS (μm)	EPITHELIUM HEIGHT (μm)
1	377,500	260,000	58,750
2	327,500	200,000	63,750
3	310,000	187,000	61,500
4	382,500	277,500	52,500
5	547,500	245,000	151,250
6	312,500	160,500	76,000
7	400,000	315,000	42,500
8	355,000	272,500	41,250
9	330,000	195,000	67,500
10	297,000	100,500	98,250
MEDIA	364,000	221,300	71,325

At the age of 180 days the medium outer diameter of the seminiferal tubules has reached the value of 364,000 μm , the diameter of the lumen inside the seminiferal tubules

has reached the medium diameter of 221,300 μm and the height of the seminal epithelium has a media of 71,325 μm (Table 2).

After the spermiogenesis process it is observed that the newly-formed spermatid differentiate themselves from the spermatozoa and the most important transformations are: the acrosome's development, the nuclear chromatin's condensation, the mobile tail's development and the loss of the spermatid's excess material (cytoplasm, water, organelles) that are not necessary to the spermatozoon and are phagocytated by the Sertoli cells later in the spermiogenesis process. In sections stained by Tanzer-Unna method interstitial gland appear in tubular tissue, delimited by elastic fibers from the basal lamina of seminiferous tubules (Figure 3).

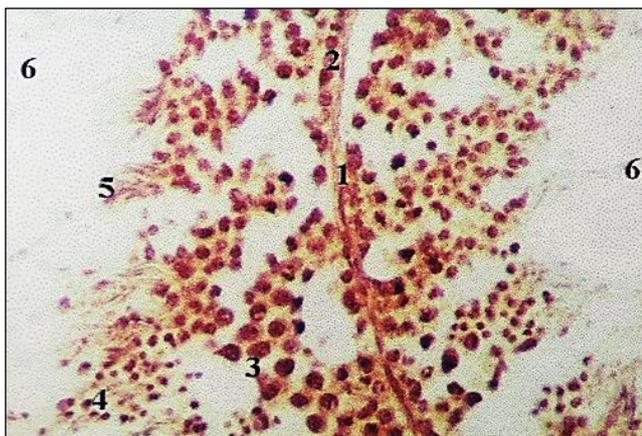


Figure 3. Seminal epithelium from two. Seminiferous tubules standing side by side in a 180 day old rooster, Tanzer-Unna, Ob. 40x.

- 1- basal membrane of the seminiferous tubule;
- 2- spermatogonia; 3- primary spermatocytes; 4- spermatid;
- 5- spermatozoa; 6- the seminiferous tubule lumen.

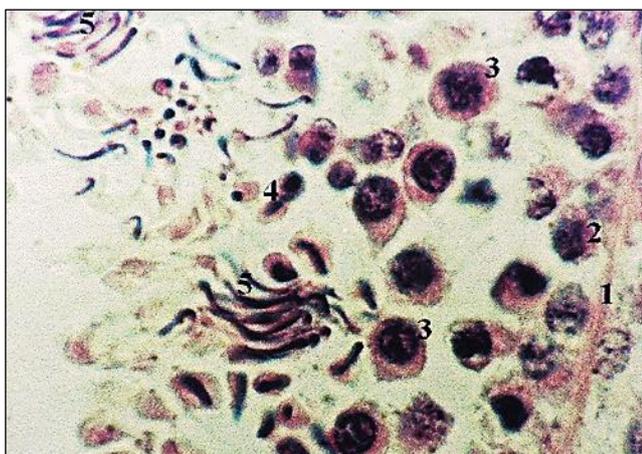


Figure 4. Spermatogenic epithelium in the rooster by 180 days, HE, Ob. 100X

- 1- basal membrane; 2- spermatogonia; 3- primary spermatocytes; 4- spermatid;

Through the electron microscopy researches done on the rooster's testicular parenchyma during its reproductive season it is observed that the Sertoli cell has a large heterochromatic nucleus with large clusters. The cytoplasm is electron-dense and with numerous spheroidal profiles of

rough endoplasmic reticulum and centriole. The myofibroblast layer is thin, sometimes there appears a myofibroblastic pluristratified component. In the Figure 5 between the fibroblasts there is a blood capillary in which it is observable the endothelium, the basal membrane of the capillary and the elements of the hemo-testicular barrier, more precise the basal membrane of the seminiferous tubule with its three components, the capillary's basal membrane and its vascular endothelium.

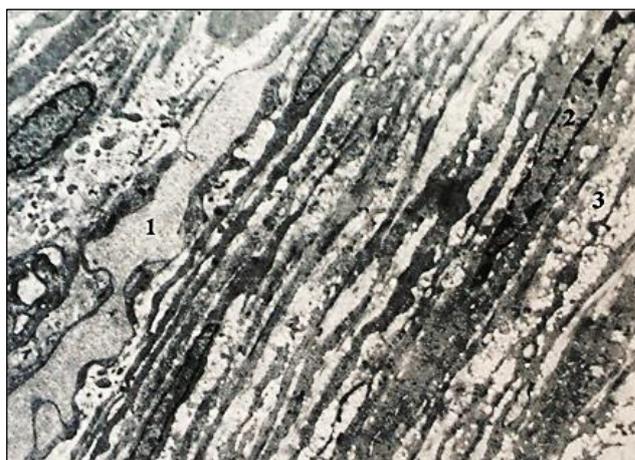


Figure 5. Basal membrane, blood capillary and intertubular space (x 8.400)

- 1- capillary; 2- myofibroblast; 3- cytoplasm with microvesicles.

Figure 6 highlights the seminal epithelium formed by a large Sertoli cell, the spermatogonia and both the 1st and 2nd order spermatocytes flanking it. In the Sertoli cells the cytoplasm is darker under the electron flux and contains numerous mitochondria, rough endoplasmic reticulum and ribosomes.



Figure 6. Seminal epithelium – ultrastructure

- 1- Sertoli cell; 2- spermatogonia; 3- 1st order spermatocyte in division; 4- 2nd order spermatocyte; 5- spermatid; 6- lysosomes.

The 2nd order spermatocyte has its chromatin arranged in a spiral this being the signal suggesting the beginning of the division.

The Sertoli cells limit the epithelium between the basal and adluminal compartments. This is possible with the help of the tight junctional complexes with a wide spread, in spot and in band desmosomes, placed between the cellular membranes of the Sertoli cells.

At a greater magnification (x 58.000) in the Figure 7 we can identify the Sertoli cell with a high nucleus in which we observe heterochromatic clusters adhering to the nuclear membrane or in the center of the nucleus. There are visible the vesicles of the smooth endoplasmic reticulum, elongated bags and ribosomes placed in a rosette.

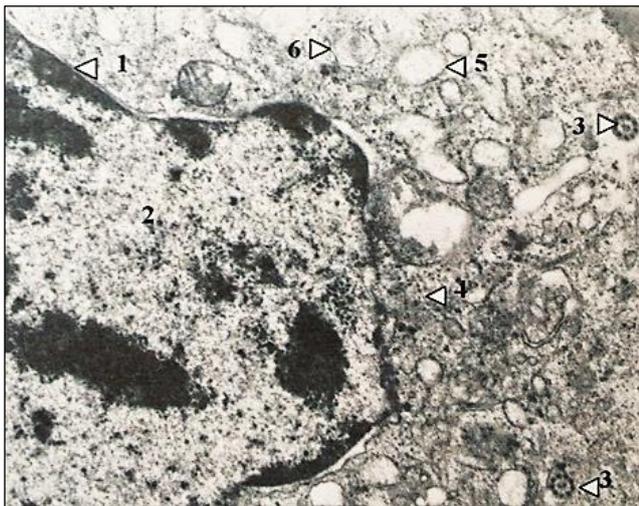


Figure 7. Seminal epithelium (x 58.000)

1-nuclear membrane; 2-nucleus with chromatin in large lumps; 3-transversely sectioned flagellum; 4-rough endoplasmic reticulum; 5-smooth endoplasmic reticulum; 6-lysosomes.

The Sertoli's cell cytoplasm is more opaque under the electron flux, it has its nucleus occupying the basal pole, numerous mitochondria and lysosomes. The Sertoli's cell cytoplasm is extended having numerous extensions to the tubule's lumen closing all the spermatogenic line's cells. The cytoplasmic limits of the Sertoli cell are very irregular and in constant modification giving permission to a progressive movement in the spermatogenesis dynamics to the lumen of seminiferous tubule.

The spermatogonia has a centralized and clustered chromatin while the spermatocyte has an euchromatic nucleus with evidenced nucleol. In the Sertoli's cell cytoplasm there are numerous observable organelles, more clearly visible being the smooth and the rough endoplasmic reticulations etc.

In the Figure 8 it is observed a 1st order spermatocyte with a large nucleus, a doubled nuclear membrane, profiles of both rough and smooth endoplasmic reticulum, large Golgi complex, free and in rosette ribosomes.

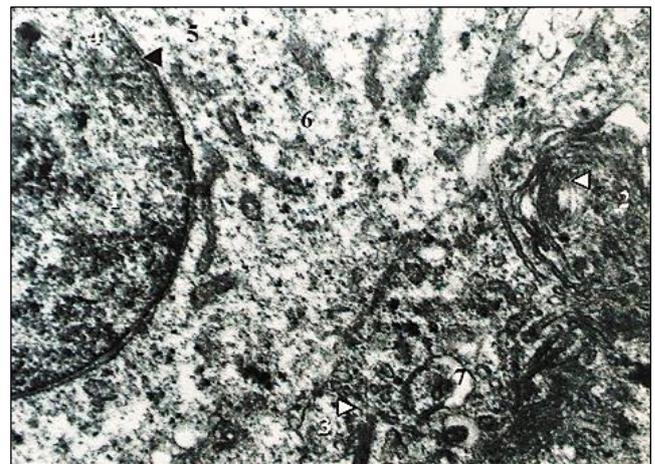


Figure 8. Seminal epithelium (x 8.400)

1- 1st order spermatocyte; 2- Golgi complex; 3- ribosomes; 4- nucleolus; 5- nuclear membrane; 6- rough endoplasmic reticulum; 7- lysosomes.

Spermatide in different phases of the spermatogenesis show up in the upper layers (Figure 9). These cells have an acrosomal vesicle in a developing state given by the large Golgi complex. To the luminal surface, the Sertoli cell covers the almost completely formed spermatozoon. The spermatid contains more abundant smooth endoplasmic reticulum and less abundant rough endoplasmic reticulum, a well-developed Golgi apparatus, numerous mitochondria and lysosomes. The newly-formed spermatozoon nuclei that were originally located centrally have become eccentrically. Shortly after the nuclear pole of the spermatozoon was found in the interior of a deep crypt inside the Sertoli cell.

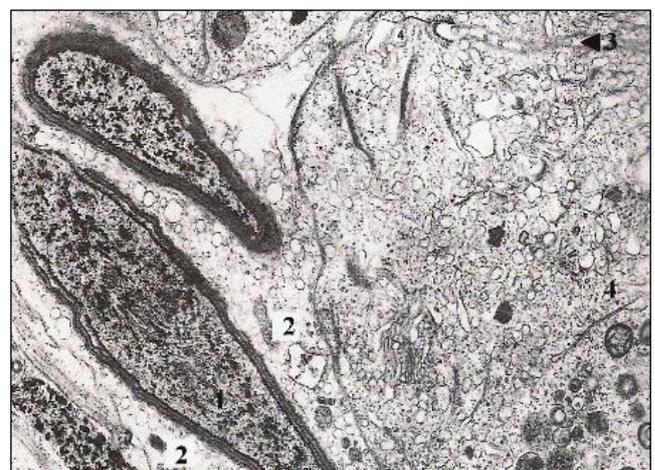


Figure 9. Sertoli cell (X10.000)

1- spermatozoon elongated; 2- Sertoli cell; 3- two plasmalemmes; 4- ribosomes.

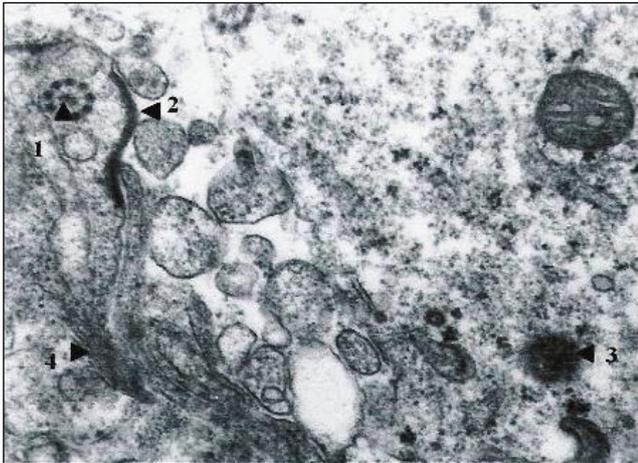


Figure 10. Seminal epithelium(X 16.500)
1- desmosomes in band; 2- lisosomi; 3- rough endoplasmic reticulum.

In the cytoplasm of the Sertoli cell appears the elongated spermatid with an ovalary nucleus and an early flagellum of the spermatozoon. There appear big junctional complexes between the Sertoli's cell cellular membrane and the membrane of the spermatocytes, junctions like desmosomes in band and elongated cisterns of smooth endoplasmic reticulum (Figure 10). Figure 11 illustrates the presence of the spermatid with flagellum. There are easily observable the limits of between the Sertoli cell and the basal membrane.

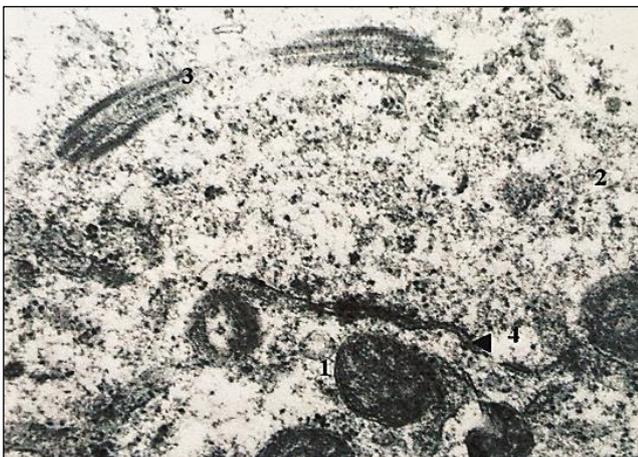


Figure 11. Seminal epithelium
1- spermatid; 2- Sertoli cell; 3- elongated spermatid's flagellum, longitudinal section;
4- junctional complexes between the Sertoli's cells membranes.

The spermatozoon's tail is made up of three segments: the proximal segment, distal segment and the terminal segment of the tail. The spermatozoon's tail is formed by the axial filament that is continued by the intermediary piece, surrounded by a thin layer of cytoplasm. the axial filament shows up being ultrastructurally formed by nine pairs of thin filaments gruped together in an identical patern

with the one existing in the cilia. In the Fig. 12 a 1st order spermatocyte is shown during the anaphase. Its chromosomes are placed in the center of the cell. In the cytoplasm several organelles are found like: smooth endoplasmic reticullum, rough endoplasmic reticullum and mitochondria.

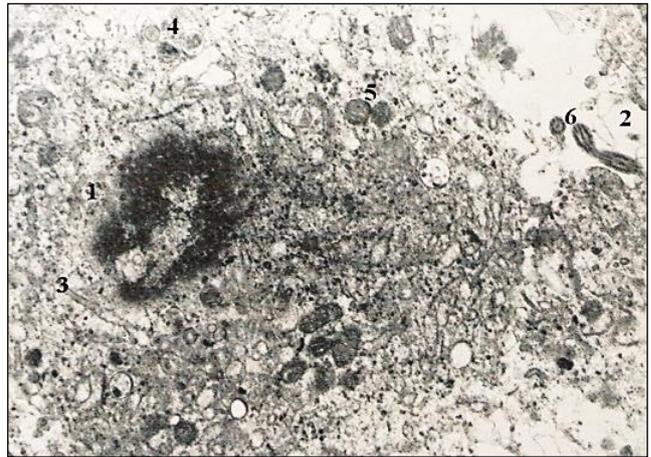


Figure 12. Seminal epithelium-spermatocyte during division (x 36.000)
1- spermatocyte, early anaphase; 2- Sertoli cell; 3- junctions between Sertoli cells; 4- lysosomes; 5- mitochondria; 6- longitudinally and transverse sectioned axoneme.

On the right side of the image is a Sertoli cell with three centrioles. During the meiotic division some small expansions of the nuclear membrane and internuclear junctions can be observed.

In the Figure 13 can be observed the areas of seminal epithelium during the stage 4 in which the late spermatid has shown up.



Figure 13. Seminal epithelium
1- nucleus; 2- nucleus of the late spermatid; 3- cytoplasm of the Sertoli cell; 4- 2nd order spermatocyte;
5- spermatocyte's nucleus with nucleolus; 6 -lysosomes.

Smooth endplasmic reticullum's cisterns are paralel with both the spermatocyte's and the Sertoli's cell cellular (Figure 14, 15).



Figure 14. Seminal epithelium (x 36.000)

- 1- nucleus; 2- Golgi apparatus; 3- nuclear membrane;
- 4- mitochondria; 5- smooth endoplasmic reticulum;
- 6- rough endoplasmic reticulum; 7- ribosomes.

The spermatid suffers a complex process of differentiation called spermiogenesis during which takes place the morphological transformation of the spermatid into spermatozoa. Before reaching the final stage, the spermatid's nuclei suffer an elongation process and reach the final phase of maturation during the spermatogenesis. Simultaneously, the centrioles migrate to the spermatid's posterior pole. The proximal centriole will make up the neck of the spermatozoon and it is formed by the proximal centriole stuck to the upper portion of the spermatozoon's head.

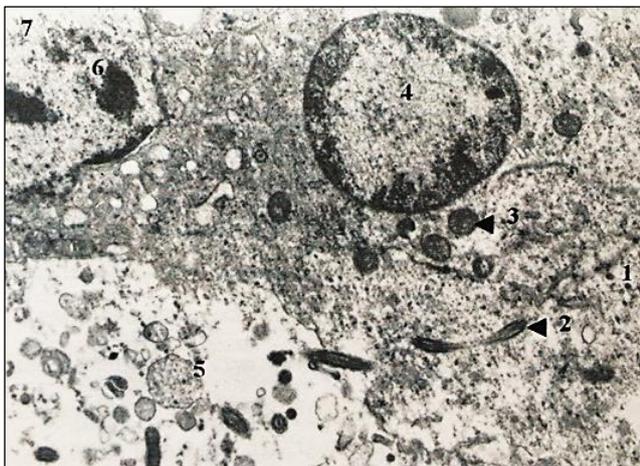


Figure 15. Seminal epithelium (x 36.000)

- 1- Sertoli's cell cytoplasm; 2- spermatid with flagellum;
- 3- mitochondria; 4- nucleus; 5- multivesicular bodies;
- 6- nucleolus; 7- spermatocyte.

Conclusion

1. At the age of 120 days in the seminal epithelium are present all types of cells of the seminal line – primary spermatocytes, secondary spermatocytes, spermatide and spermatozoa, that indicating onset of spermatogenesis

process. Sertoli cells were observed in cross sections of seminiferous tubules, they are willing unistratal, with the nuclei located basal, polymorphous nucleolus sometimes triangular course.

2. Sertoli cell is located on the basement membrane of seminiferous tube that makes complex junctionale.

3. At the age of 120 days the interstitial Leyding gland appears in intertubular tissue, delimited by the elastic fibers from the basal lamina of seminiferous tubules.

4. It is noted that the basement membrane of seminiferous tube is thick and shows numerous invagination directed to the cytoplasm of the Sertoli cells.

5. The cytoplasm of the Sertoli cell is dense and has more organismous – numerous cistern of smooth endoplasmic reticulum, numerous mitochondria, rough endoplasmic reticulum profiles, lysosomal, Golgi complex, centrioli, etc.

6. The electronmicroscopy studies done during the reproductive season have highlighted the elements of the hemo-testicular barrier made up by the lamina basalis of the blood capillary, capillary endothelium and the capillary's lumen.

7. The intersertolian junctions at the level of both lateral and apical cellular membranes of the Sertoli cells have junctional complexes(tight, desmosomes in band, GAP). These mark the limits of the basal and adluminal compartments.

8. Early spermatids with a spheroidal shape from the seminal epithelium in the rooster present many passing phases to the last stage of spermatozoon and initial spherical nucleus of the spermatid gets elongated.

9. From the initial spheroidal spermatid's cytoplasm there appears a longitudinal cytoplasmatic cuff, aspect that represents the pass to the late spermatid stage.

10. In the roosters appear modifications of length and thickness of the centriole's wall and simultaneously they migrate to the posterior pole of the spermatid.

11. In the stage of late spermatid there will be observed 3-4 dense extensions in the space of the nuclear invagination and the presence of dense bodies next to the distal centriole

12. The mitochondria arranges itself around the axonema in the middle piece and appears the dissolution of the cytoplasmic cuff with the help of the Sertoli cell.

13. In which it concerns the morphometric dynamics of the exterior diameter of the seminiferous tubules, this has grown with the ages so that the medium outer diameter at the age of 120 days was 217,575 μm , while at 180 days it 364,000 μm .

14. The medium diameter inside the seminiferous tubule's lumen also presents variables with aging. So, at the age of 120 days the diameter was 104,716 μm , while at 180 days it was 147,900 μm .

15. The height of the seminal epithelium has presented morphometrical variations with aging and the spermatogenetic wave. At the age of 120 days the medium was 56,429 μm , while at the age of 180 days it was 71,325 μm .

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