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# CIRCANNUAL CYCLE OF THE SERTOLI CELL NUCLEOLUS IN ROE DEER, *CAPREOLUS CAPREOLUS*, AND SEASONAL CHANGES OF ITS LEYDIG CELLS: AN ULTRASTRUCTURAL STUDY

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**Ethical considerations:** When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

## ABSTRACT

**Ruminant Sertoli cells, instead of a nucleolus with nucleolonema, have a vesicular nucleolus, first described as a multivesicular nuclear body (MNB). It consists of membrane vesicles and tubules covered with ribosome-like granules. We studied the testes of several domestic and wild ruminants using transmission electron microscopy. In domestic ruminants with continuous spermatogenesis, the MNB occurs throughout the year. In roe deer, the seasonal breeder, the most developed MNB is during the rut. During testicular rest, Sertoli cells have a nucleolus without vesicles. The cycle of Sertoli cells nucleolus is related to the onset and cessation of spermatogenesis and cyclical changes in Leydig cell, which include their regression, apoptosis and autophagy. Before and during spermatogenesis, Leydig cells have all the features of steroid-producing cells. At the end of the rut, the amount of smooth endoplasmic reticulum (SER) decreases rapidly and lipid droplets and glycogen appear in their cytoplasm. In January, small inactive Leydig cells contain lipid droplets, remnants of SER and glycogen. Our observations indicate that the MNB performs a major function of the nucleolus – a key role in ribosome biogenesis. Why membrane vesicles are present in the MNB, and what their role is in ruminant Sertoli cells, remains a mystery more than 50 years after its discovery.**

**Key words: Leydig cell; roe deer; Sertoli cell nucleolus cycle; ultrastructure**

## INTRODUCTION

Sertoli cell, also termed sustentacular, supporting, or "nurse" cell, *Sustentocytus testis,* also *Epitheliocytus sustentans,* is a unique cell. It has more functions than any other among over 200 cell types of the body does, making it a unique cell necessary for spermatogenesis [1, 2, 3, 4, 5, 6]. Apart from support, protection and nutrition of

developing germ cells via various signal pathways [3, 7, 8], Sertoli cells have other functions crucial for spermatogenesis [5, 9, 10, 11]. They form a blood-testis barrier – BTB [12, 13, 14, 15, 16, 17, 18], and transport nutrients and many regulatory factors through it to developing germ cells and hereby create a specific environment necessary for spermatogenesis [2, 3, 19, 20]. The BTB physically divides the seminiferous epithelium into basal and apical (or adluminal) compartments [13, 15, 16, 17] and is pivotal to spermatogenesis [2, 3, 20, 21]. Sertoli cells communicate with each other via gap junctions [22, 23, 24, 25]. Sertoli cells are phagocytic [26], they remove residual bodies [27, 28], phagocytose degenerated [29, 30] and apoptotic germ cells [31, 32]. Phagocytosis is morphologically the most noticeable function of Sertoli cells [33], and is an essential event for spermatogenesis [10]. Sertoli cells also play a role in the transport of germ cells across seminiferous epithelium [34, 35] and in spermiation – the release of sperms from seminiferous epithelium [3, 36, 37, 38, 39, 40, 41]. Sertoli cells also secrete isosmotic fluid, which carries immotile testicular sperms from their site of origin to the rete testis and excurrent ducts [9, 42, 43, 44]. Specific Sertoli cells in the tubuli recti form a plug-like structure, serving a valve preventing the reflux of fluid from the rete testis into the seminiferous tubules [45, 46, 47, 48]. Sertoli cells are polarized; showing bidirectional both endocrine and exocrine [49] as well as paracrine [2, 6, 50, 51, 52, 53] and autocrine activity [2, 11, 54, 55, 56]. They produce anti-Müllerian hormone – AMH [57, 58, 59], inhibin [60, 61] and activin [56] and androgen-binding protein – ABP [62, 63, 64, 65]. Sertoli cells also secrete many regulatory growth factors, which play a key role in local regulation of spermatogenesis [2, 8, 52, 54] and Leydig cells [53, 66,]. The secretory products of Sertoli cells are pivotal in fostering germ cell development and directing the appropriate maturation of sperm [11]. Creation of BTB [5, 13, 18] and secretion of numerous factors necessary for normal development of germ cells [8, 52, 54, 55] are the two basic functions of Sertoli cell, which are essential for spermatogenesis [2, 4, 19, 21]. Through them, Sertoli cells create and control the special environment within the seminiferous tubules necessary for the progression of germ cells to spermatozoa [2, 3, 16]. In addition to their essential role for germ cell development, Sertoli cells also play a role in regulating androgen production in the testes [53, 66]; all of this makes Sertoli cells "key drivers of testicular function" [5]. Sertoli cells also form unique tubulobulbar complexes around spermatids [67, 68, 69, and others], which are cytoskeleton-related structures indispensable for spermiation [34, 41].

The wide range of functions of Sertoli cell is reflected in its diverse structure and vice versa. There is no other cell in the body with such a diverse structure as the Sertoli cell. The ultrastructure of mammalian Sertoli cells has been reviewed by many authors [1, 27, 70, 71, 72, 73, 74, 75, 76]. Unusual characteristics of Sertoli cells include a nucleolus containing membrane vesicles, which is found only in ruminant Sertoli cells. First, Nicander et al. [77] described the ontogenetic development of vacuolated nucleolus in "indifferent cells" in seminiferous tubules of three-and-a-half to five-and-a-half-month old bull calves. Then Nicander [78] was first to observe "vacuoles within and around the nucleolus" of the bull and ram Sertoli cells. Zibrin [79] published the first electron micrographs of the Sertoli cell´s unique nucleolus of normal adult bulls and was the first to describe in detail this structure consisting of membrane vesicles covered with ribosome-like granules as a multivesicular nuclear body (MNB) with nucleolar activity. This was confirmed by all who later studied the Sertoli cells of the bull under an electron microscope [46, 47, 70, 72, 74, 80, 81, 82, 83, 84, 85, 86, 87]. The MNB, later also called vesicular nucleolus, was found in Sertoli cell in other domestic and wild ruminants such as ram [46, 70, 85, 88], gerenuk [70], chamois [86], Cape buffalo [70], roe deer [88], water buffalo [90, 91, 92], lesser mouse deer [93], West African dwarf goats [94, 95, 96], Shiba goat [97, 98] and Egyptian Nubian goat [99]. The only known exception so far is the fallow deer, *Dama dama;* its Sertoli cells have a reticular nucleolus with nucleolonema [74, 89, 100] instead of the MNB - vesicular nucleolus.

In this retrospective paper, we summarize our 25-year previous study of the Sertoli nucleolus in various ruminants, focusing on the structure of the roe deer Sertoli cell nucleolus throughout the year as well as the ultrastructure of its Leydig cells. In addition to the results of our electron microscopic observation, we present a minireview of Sertoli cell structure and function. We also include the short history of the discovery of the MNB, later also called the vesicular nucleolus, because some reviews of Sertoli cells did not respect it or just simply ignored it [72, 73, 76]. More than half a century has passed since its discovery [78, 79] and it is still not known what causes its develop-

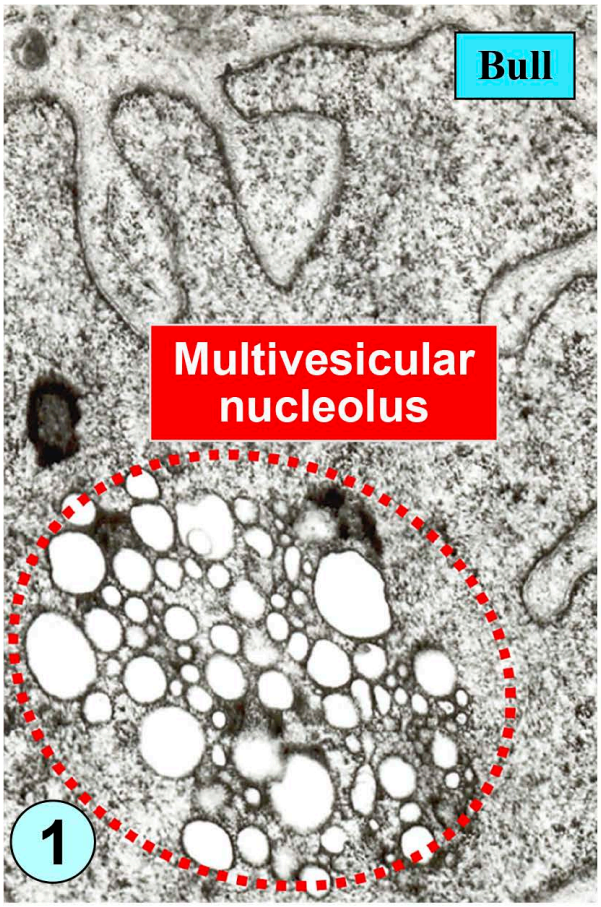
ment, what its function is, why MNB is found in Sertoli cells and why only in ruminants. Therefore, one more goal of this paper is to draw attention to further study of MNB in Sertoli cells of ruminants.

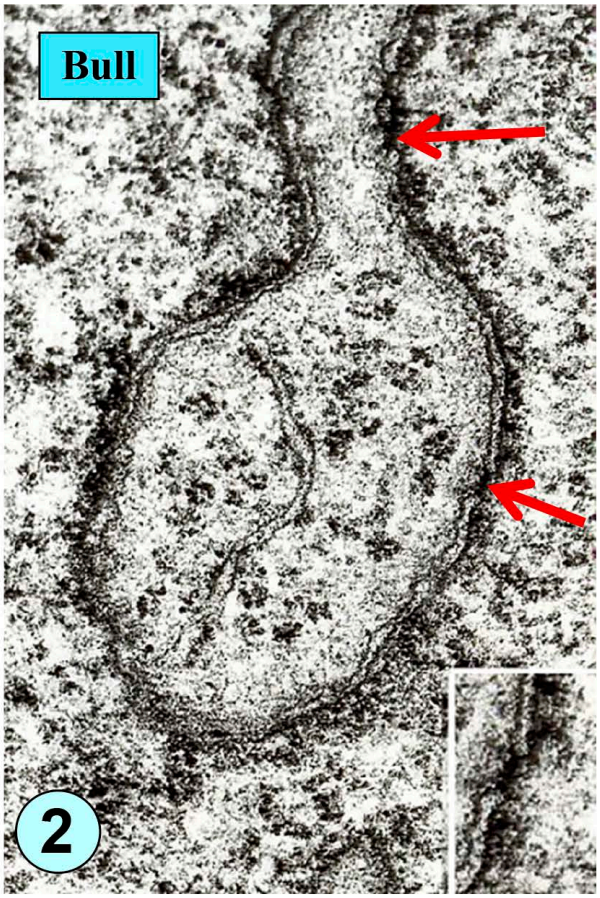
## MATERIAL AND METHODS

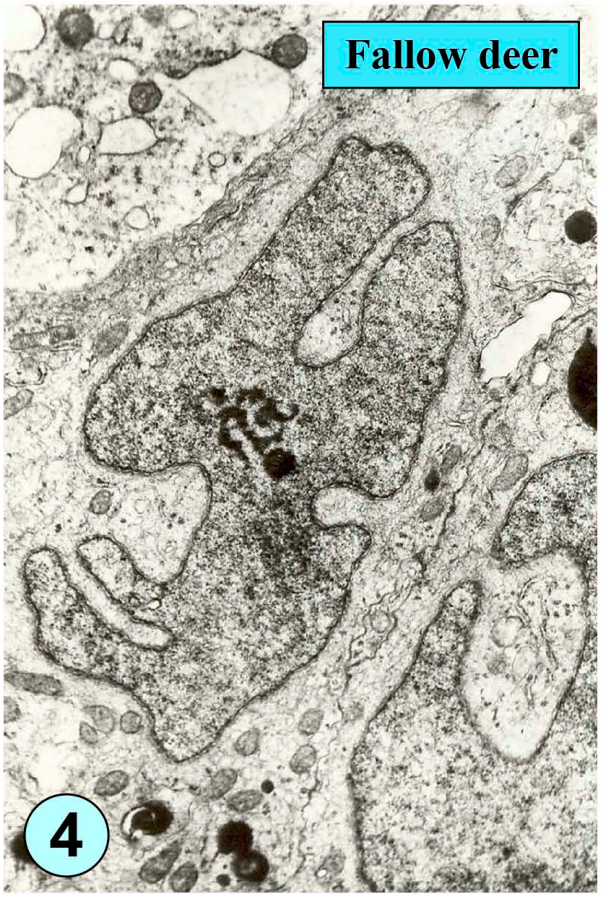
We studied testes of roe deer *(Capreolus capreolus),* fallow deer *(Dama dama),* bull*,* ram and goat and several other domestic mammals such as the stallion, boar, and dog and rodents mouse and rat by conventional transmission electron microscopy (TEM). We obtained the testes of seven roe deer 2- to 7-years-old shot on January 1, April 15, mid-June, mid-July and the end of August, testes of a 9-month-old fallow deer and of four 2- to 7-year-old fallow deer shot in heat in October - November. Eight bull testes samples we collected from 2-year-old healthy adult bulls slaughtered at a local slaughterhouse. The testes of two adult rams and a goat we collected from animals used for anatomy dissections in our department. Small, 1-3 $mm^3$ pieces of tissue were immediately fixed in 3% glutaraldehyde (Sigma-Aldrich, Bratislava, Slovak Republic) in 0.15 M cacodylate buffer pH 7.2 for 3 hours, or in a mixture of 2.5% paraformaldehyde and 2% glutaraldehyde, both in 0.15 M phosphate buffer solution pH 7.2–7.4 and followed by 1% $OsO_4$ (Fluka Chemie AG, Buchs, Switzerland) in the same buffer for 2 hours. Rinsed samples, rapidly dehydrated in acetone, we further processed routinely and embedded in Durcupan ACM (Sigma-Aldrich Chemie GmbH, Germany). Since the first observations of bull Sertoli cell and its nucleolus of the first author date back to the late 60s of the last century, he fixed his first bull testis samples in 3% glutaraldehyde in 0.1 M phosphate buffer with 5% sucrose pH 7.4–7.6 for 2 hours each. Fixed samples were rapidly dehydrated in alcohol followed by propylene oxide and routinely embedded in Araldite or Durcupan ACM. We cut ultrathin sections on the TESLA BS 490 ultramicrotome using glass knives, double stained with uranyl acetate and lead citrate (Merck, Bratislava, Slovak Republic) and photographed in electron microscopes Tesla BS 500, JEM 1200 EX and JEM-100 CX (JEOL, Japan). From all testes, immediately after sampling for TEM, we took samples for histological examination under a light microscope (LM). We fixed slices of testis 3 to 5 mm thick in 4% neutral formaldehyde in 0.1 M phosphate buffer pH 7.2–7.4 and processed routinely. Paraffin sections 3-5 μm thick were stained with hematoxylin and eosin (H&E).

## RESULTS

The largest and best-developed MNB among the Sertoli cells of ruminants is in the bull. The typical MNB also known as the vesicular nucleolus of bull Sertoli cells as in Fig. 1 consists of membrane vesicles of various sizes and a few tubules, both covered with ribosome-like granules and a small amount of fibrillar material. In all non-ruminants, we observed a reticular nucleolus with nucleolonema, and no membrane vesicles. We did not observe any seasonal changes in the nucleus and nucleolus in the bull Sertoli cell; the MNB – vesicular nucleolus is found throughout the year. The Sertoli cell's nucleus in all ruminant species has a folded surface, its nuclear envelope forms numerous deep invaginations towards the MNB. Invaginations contain ribosomes and occasionally one or two cisternae of rough endoplasmic reticulum (Fig. 2). The structure of the Sertoli cell of the roe deer and its nucleolus during spermatogenesis and in rut (Fig. 3) looks like that of other ruminants, including the MNB of the bull and other ruminants except the fallow deer, *Dama dama* (Figs. 4, 5). Sertoli cells of fallow deer also have a euchromatic nucleus with a folded surface and deep invaginations of nuclear envelope that are full of ribosomes (Fig. 4), but instead of a vesicular nucleolus, they have a reticular nucleolus with a nucleolonema (Fig. 5). We found such a more or less similar nucleolus with a nucleolonema in Sertoli cells of dog, cat, fox, stallion, boar, mouse, and rat. Rat and all rodent Sertoli cells have a tripartite nucleolus with a prominent reticular nucleolus flanked by two round, electron-dense heterochromatic bodies. The roe deer is a seasonal breeder. Its spermatogenesis begins in March - April. Sertoli cells are activated at about the same time, or a little earlier. Many mostly small vesicles appear in the nucleolonema of fibrillar material and 1-2 large vesicles are found on the edge of the reticular nucleolus (Fig. 6). The largest, best-developed MNB of roe deer Sertoli cell with the most numerous large round vesicles occurs during the rut from mid-July to mid-August. The nucleolus mainly consists of large and smaller vesicles (Fig. 7) and is quite similar to the MNB of bull Sertoli cells. Unlike the bull, a web of fine filaments covers most of the vesicles. In July, some of the


Bull
Multivesicular nucleolus
1


Bull
2


Fallow deer
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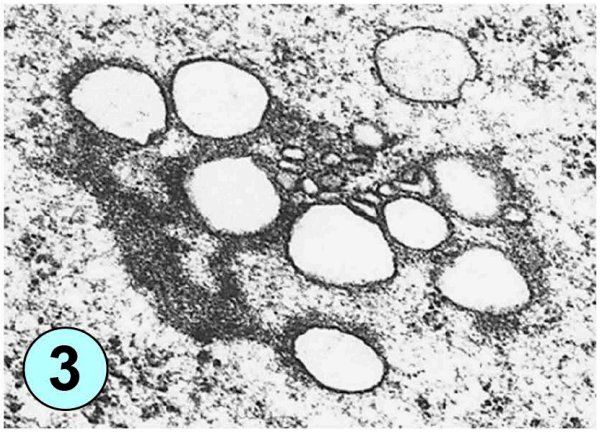
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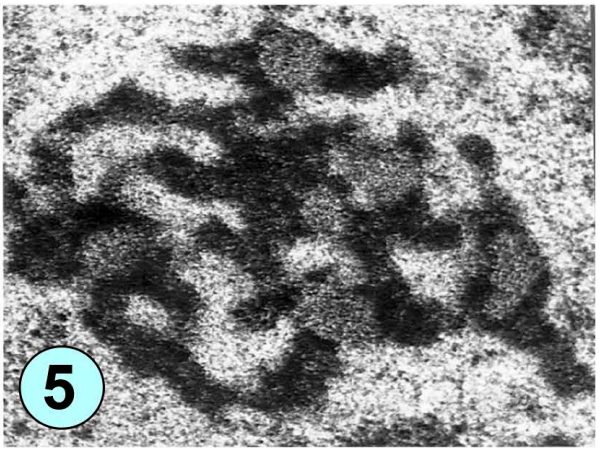
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**Fig. 1. Multivesicular nucleolus – a typical multivesicular nuclear body (MNB) of a bull Sertoli cell. The nuclear envelope forms numerous folds towards the MNB. 9,500 x.**
**Fig. 2. Close-up view of invagination of the nuclear envelope of a bull Sertoli cell with many free polyribosomes and a few membrane-bound to RER cisterna. Nuclear pores (arrows) and in an inset, and perinuclear vimentin filaments are also clearly visible. 60,000 x; inset 90,000 x.**
**Fig. 3. Vesicular nucleolus with a rudimentary nucleolonema of Sertoli cell of the roe deer in rut when the MNB in this seasonal breeder is the largest. The roe deer shot in July. 30,000 x.**
**Fig. 4. Sertoli cell of the fallow deer with an elaborated nucleus and a reticular nucleolus. Large invaginations of nuclear membrane contain free ribosomes and a few profiles of RER cisternae. The fallow deer was shot during rut in October. 8,000 x.**
**Fig. 5. Reticular nucleolus with nucleolonema in Sertoli cell of the fallow deer in rut. The fallow deer is the only so far known exception among ruminants, in which no large, round vesicles are found in the nucleolus of its Sertoli cells in active testis or during the rut. 33,500 x.**

vesicles, mostly large ones, were partially covered with ribosome-like granules. The nucleolonema is barely visible. In August, when the rut ends, spermatogenesis also ends. Residual bodies, which are remnants of phagocytosed dead or apoptotic spermatogenic elements and sperms, we found in the Sertoli cells. In January, the testis is at its most dormant. The outline of the nucleus of Sertoli cells smoothed, there are almost no invaginations of the nucleus envelope. Sertoli cells have a reticular nucleolus with a dense distinct nucleolonema (Fig. 9). Vesicles in the Sertoli cells' nucleoli have almost completely disappeared (Fig. 10), we observed 1-2 small vesicles in the nucleolonema of about every fourth nucleolus of Sertoli cell. Spermatogenesis does not take place. The seminiferous epithelium consists of Sertoli cells (Fig. 11) and spermatogonia, but in some seminiferous tubules there were few spermatocytes in addition to spermatogonia. No advanced stages of spermatogenesis such as round or elongated spermatids were present (Fig. 11).

We also observed seasonal changes in the ultrastructure of Leydig cells that corresponded to their varying steroidogenic activity during the year. In January, at the maximum rest in the testis, Leydig cells were small, had an irregular shape and contained lipid droplets (Figs. 12, 14, 15), membranous whorls of SER residues (Figs. 13–15), various dense bodies – the end products of lysosomal degradation (Fig. 13) and a small amount of glycogen (Figs. 13, 14). We also found autophagosomes with membrane whorls of residual SER, lipid droplets, and mitochondria in many inactive Leydig cells in January (Figs. 12, 14). Some autophagosomes contained remnants of glycogen, most of the glycogen was free in aggregates in the cytoplasm (Fig. 14). These were residues of glycogen, which we first observed as round glycogen aggregates together with fat droplets and rapid decrease of SER volume in Leydig cells in August (Fig. 16), which causes the well-known sudden drop of testosterone levels. In August and January, we also observed chromatin margination of Leydig cells and nuclear fragmentation in some cells, both typical of apoptosis. It is noteworthy that in mid-August, at the end of heat, the vesicular nucleus of Sertoli cells is still well developed (Fig. 17), but in some places already there is some nucleolonema with tiny vesicles or tubules (Fig. 17, arrows), typical of the reticular nucleolus at the beginning of the cycle after the testicular quiescence in January. A detailed description of seasonal changes throughout the year in the structure of roe deer Leydig cells will be the subject of a separate paper.

## DISCUSSION

The nucleolus, the most prominent structure in a cell nucleus, is a membrane-less intranuclear organelle involved in ribosome biogenesis and protein synthesis. Its primary functions are ribosomal RNAs synthesis, processing, and assembly of ribosome subunits in ribosomes biogenesis [101, 102, 103, 104, 105, 106 and others]. As a result, the nucleolus is a necessary part of the proteosynthetic apparatus of the cell [107, 108, 109 and many others]. Early studies of Sertoli cells identified that they synthesize and secrete over 60 proteins, a very diverse range that includes the hormones inhibin B, anti-Müllerian hormone, activin, transport proteins like androgen-binding protein (ABP), transferrin, ceruloplasmin and testibumin, several growth factors e.g., clusterin, proteins with enzymatic activities such as plasminogen, cytokines e.g., stem cell factor (SCF), and others [8, 54, 55, 56, 110, 111, 112]. More recent proteomic and genomic analyses identified that Sertoli cells synthesize hundreds of different proteins and polypeptides, which are essential for supporting spermatogenesis and regulating testicular functions [113, 114]. This requires that Sertoli cells must have an efficient proteosynthetic system first decoding a large part of a cell genome and including an active nucleolus producing ribosomes. Indeed, the synthesis of a large number of differ-

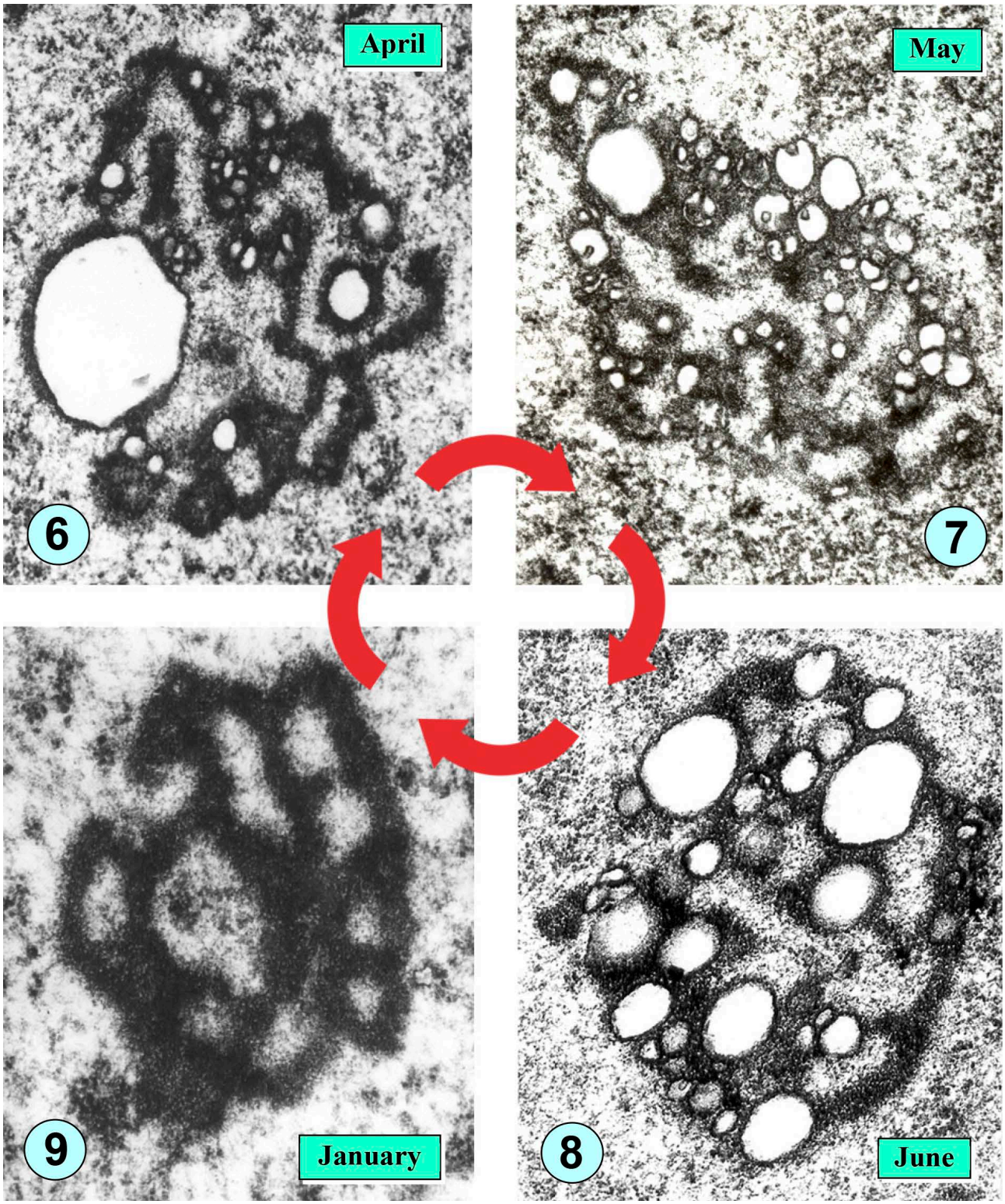


**Fig. 6. Vesicular nucleolus of a Sertoli cell at onset of spermatogenesis. April. 28,000 x.**
**Fig. 9. Reticular nucleolus of a Sertoli cell of roe deer in January. 30,000 x.**

**Fig. 7. Vesicular nucleolus of a Sertoli cell of roe deer before the rut in May. 18,500 x.**
**Fig. 8. Part of vesicular nucleolus of Sertoli cell in June; roe deer shot in rut. 32,000 x.**

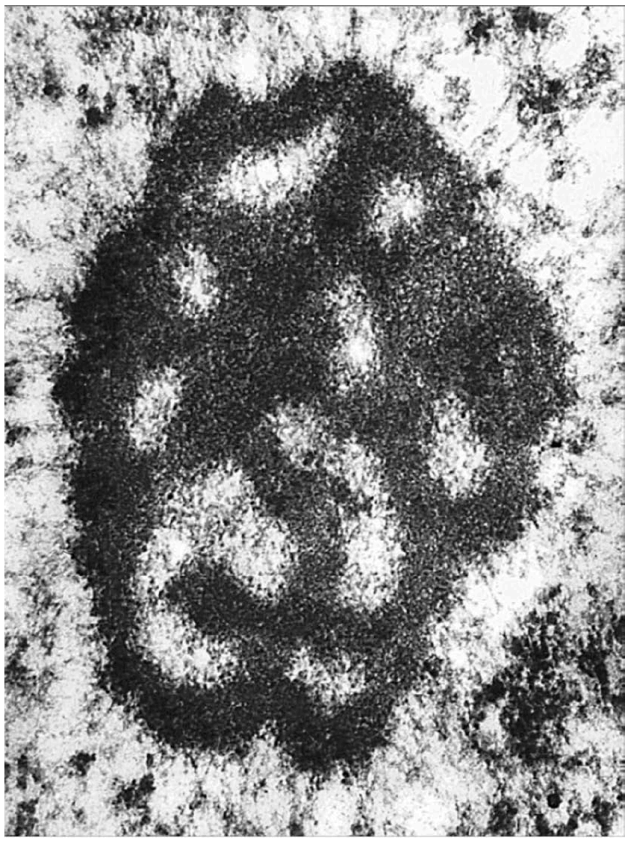

Fig. 10. Reticular nucleolus with a distinct nucleolonema of Sertoli cell of roe deer in January. It consists mainly of fibrillar part, granular compartment is small. 32,000 x.

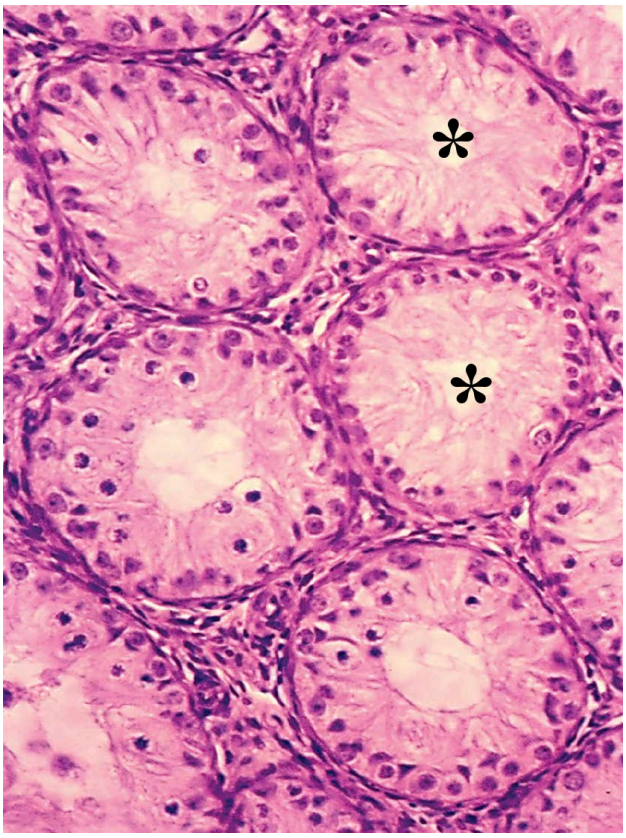

Fig. 11. Histology of the quiescent testis of a roe deer in January. No developing germ cells are in seminiferous tubules marked by an asterisk, not many are in others. LM 400 x.

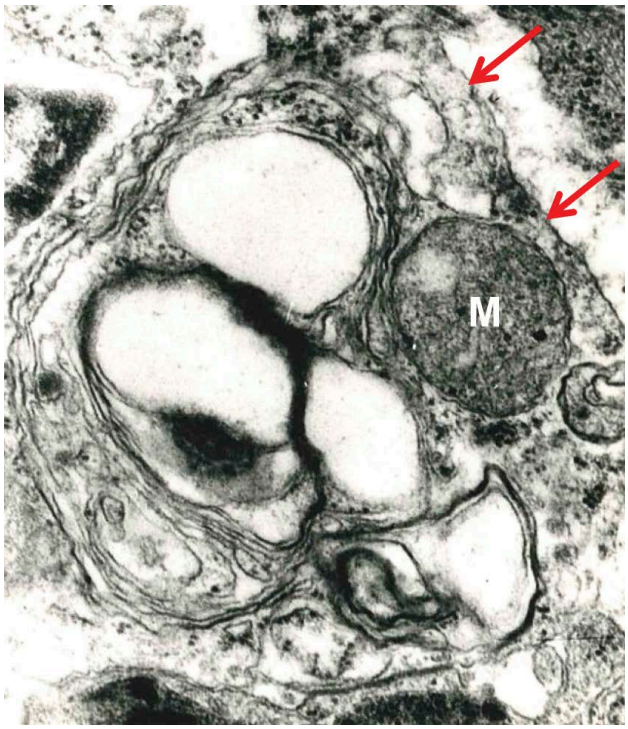


Fig. 12. Autophagosome in inactive Leydig cell with the membranous whorls of residual SER and mitochondrion (M). 29,600 x.

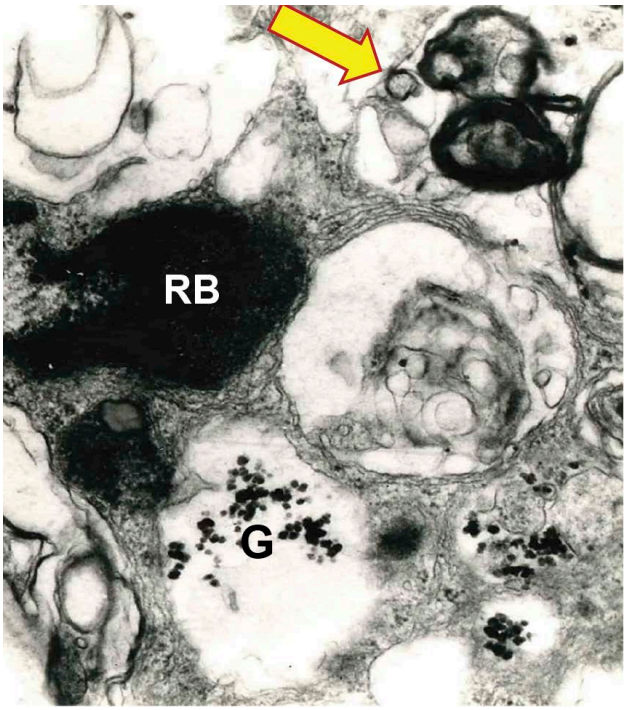


Fig. 13. Detail of an inactive Leydig cell in January. Note the residual SER (arrowhead), glycogen (G), residual body (RB). 29,600 x.

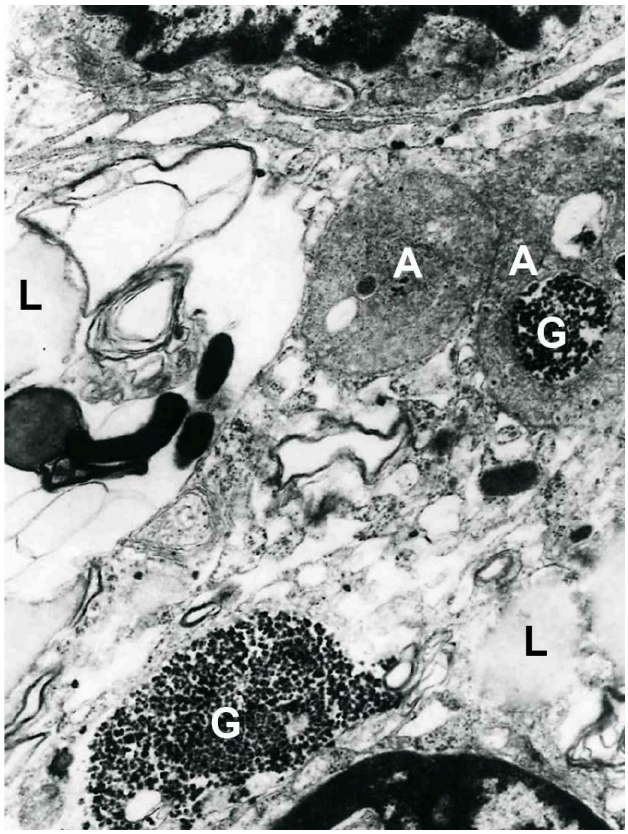


Fig. 14. Autophagic vacuoles (A), one with glycogen (G), lipid droplets (L), SER and glycogen in non-active Leydig cell. 16,600 x.

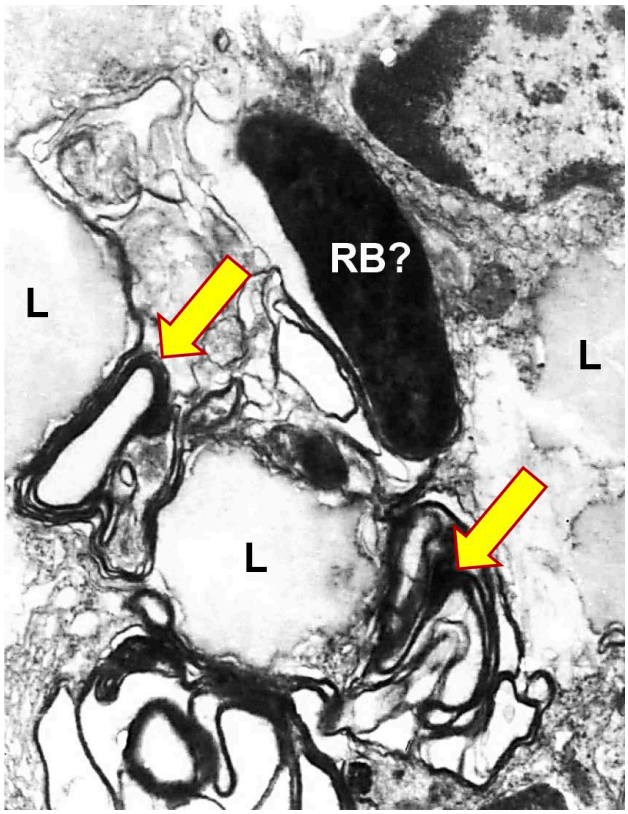


Fig. 15. Detail of non-active Leydig cell. The myelin-like whorls of residual SER (arrows), lipid droplets (L), residual body (RB). 20,200 x.

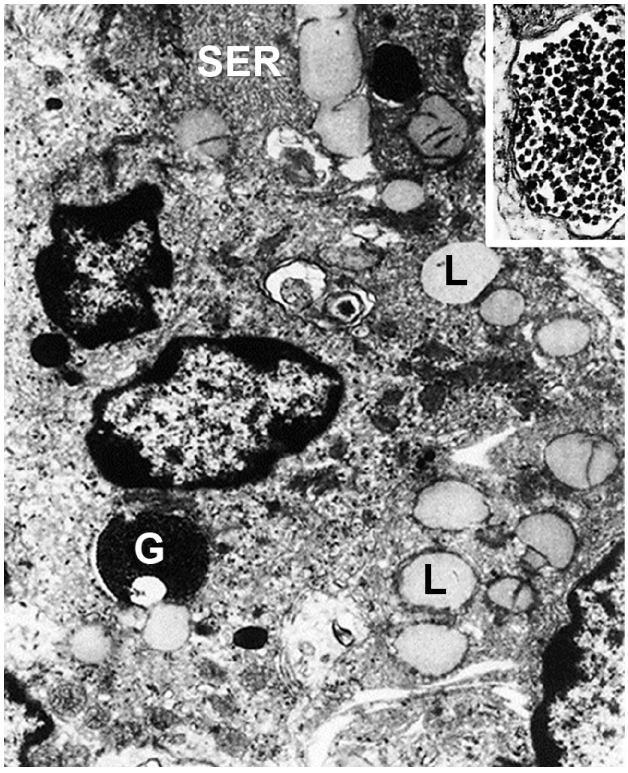


Fig. 16. Leydig cell at the end of rut. Some SER still present, several lipid droplets (L), glycogen (G), marginated chromatin. Mid-August. 9,000 x. Inset Glycogen at 20,200 x.

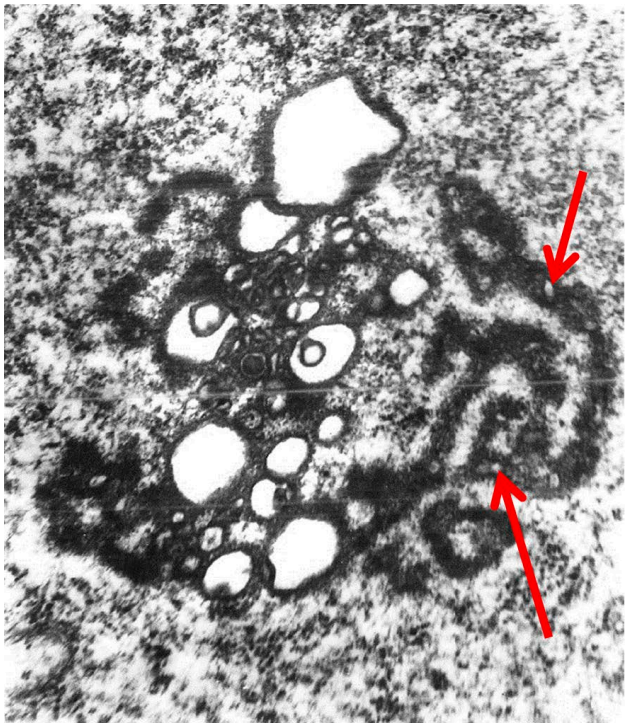

Fig. 17. Large vesicular nucleolus of Sertoli cell with a small nucleolonema and very small vesicles and tubules in it (arrows) at the end of rut. Roe deer shot in Mid-August. 19,500 x.

ent proteins and polypeptides in Sertoli cells corresponds to their structure/ultrastructure. And indeed, Sertoli cells have a very euchromatic nucleus with a prominent species-specific nucleolus. In most species, the nucleolus is reticular with a distinct nucleolonema, which in rodents is flanked by two round, dense heterochromatic satellite karyosomes. While the euchromatic nuclei of the other three most euchromatic cells in the body: nerve cells, hepatocytes and blastomeres are round, the nuclear surface of the adult Sertoli cell is folded, and their nuclear envelope numerous forms numerous deep invaginations towards nucleolus, and towards the MNB in ruminants. The nuclear envelope in invaginations has numerous nuclear pores; invaginations expand and contain many ribosomes.

One of the main characteristics of nucleolus is that they do not have membranes. The nucleolus of ruminant Sertoli cells consisting of membranous vesicles is unique; there is no other analogue of it in the animal kingdom. The only other exception is the nucleolar channel system (NCS) in epithelial cells of human endometrium [115, 116, 117, 118, 119, 120, 121 and many others]. NCS is composed of several rows of coiled tubules embedded in an amorphous matrix, the outer surface of the tubules are numerous electron-dense particles resembling ribosomes [117, 118, 119, 120, 121, 122]. The progesterone dependence of NCS in the human endometrial epithelial cells was found shortly after its discovery [115, 116, 117] by Kohorn et al. [123]. Currently NCS is a well-established ultrastructural hallmark of the post-ovulation mid-secretory human endometrium, and a potential marker of uterine receptivity [121, 122, 124, 125, 126]. Its transient presence has been associated with fertility [122, 126, 127, 128, 129], but its presence or absence cannot serve as a predictor of infertility [126]. On the other hand, a delay or absence of NCS formation in endometrial cells has been found in some infertile women with unexplained sterility [130, 131, 132]. Dockery et al. [132] observed its impaired development in women with unexplained infertility with a relative excess of luteal estrogen. Guffanti et al. [124] found that NCSs contain proteins of the nuclear pore complexes, inner nuclear membrane, nuclear lamina and endoplasmic reticulum. They used monoclonal antibodies, phase contrast and double fluorescence microscopy, which allows detection of NCS by light microscopy, a great technique to recognize NCS in endometrium and exploit for reproduction without electron microscopy. This will allow applying theoretical knowledge about NCS in practice even despite that the composition and function of the membrane system of NCS have not yet been fully clarified [121, 128, 129]. However, less than a decade after the discovery of NCS [115, 116, 117], Kohorn et al. [123] already demonstrated in vitro that NCS formation is caused by progesterone and subsequent research has confirmed that NCS formation depends on a specific progesterone threshold [127, 128, 133 and others]. On the other hand, more than half a century has passed since the discovery of the Sertoli cell MNB of the bull [79], and we still do not know what causes its development and why. It is still unknown whether its production depends on follicle-stimulating hormone (FSH) or another hormone and why MNB only occurs in ruminants. It is worth noting that MNB – vesicular nucleolus of Sertoli cells in ruminants has been included in veterinary histology textbooks [134, 135]. In contrast, NCS in the women´s endometrium is a clinically significant marker of endometrial receptivity and the "window of implantation" [122, 123, 124, 128, 129] relevant to reproduction, but despite this, no human histology textbook mentions it yet.

Andriana et al. [98], in their thorough study of the postnatal development of the MNB in the Sertoli cell of Shiba goats, found that it arises as narrow channels from the inner membrane of the nuclear envelope, gradually enlarges and moves from the periphery to the central region. We did not find such successive stages of MNB development as Andriana et al. [98] because its seasonal development in adult roe deer is probably faster. Nevertheless, it is very likely that it develops in the same way in roe deer and all ruminants except fallow deer, which does not have MNBs. A reticular nucleolus is present in Sertoli cells of fallow deer throughout the year, even in adults [74, 89, 100]. MNB is stunted or less developed in some breeds of goat [95, 96] and sheep [85]. Due to the large number of vesicles in Sertoli cells in adult bulls and most other ruminants, we consider multivesicular nucleolus – MVN to be a more appropriate name instead of vesicular nucleolus.

All authors who studied Sertoli and Leydig cells in seasonal breeders [136, 137, 138, 139 and others] found significant changes in their structure throughout the year. Both cells in the testes undergo significant ultrastructural changes throughout the annual reproductive cycle. Spermatogenic arrest results in the accumulation of lipids and primary and secondary lysosomes in Sertoli cells [99, 137, 138, 139]. Also worth noting is the change in nuclear shape in prepubertal versus adult and in inactive versus active

Sertoli cells. The nuclei of prepubertal Sertoli cells and inactive Sertoli cells of seasonal breeders of the quiescent testis are round or oval with a smooth surface; and if they have few invaginations, these are wide and shallow. Cameron et al. [140], in their model experiments on rats, found that FSH causes invaginations of the nuclear envelope of Sertoli cells containing a large number of polyribosomes. The nuclei of adult Sertoli cells in domestic ruminants and Sertoli cells of seasonal breeders before and during spermatogenesis also have several deep invaginations towards the MVN. Near the MVN, the invaginations widen and contain many ribosomes. All the above suggest that the MVN, like the reticular nucleus with nucleolonema, has a key role in ribosome biogenesis.

Simultaneously with changing roe deer Sertoli cell structure and nucleolar cycle, we observed circannual seasonal changes in the structure of Leydig cells. Their main function is secretion of testosterone, necessary for spermatogenesis and hence fertility [66]. The best structural measure of the endocrine activity of Leydig cells and testosterone production is the volume of their smooth endoplasmic reticulum (SER) [28, 141, 142, 143, 144]. We found substantial changes in SER volume of roe deer Leydig cells throughout the year, which responded to changing testosterone blood/serum levels found by [145, 146, 147, 148]. Testosterone production in roe deer shows a biphasic pattern. There is mild increase in April; the highest testosterone levels occur during the peak of the rutting season in mid-July in mid-August, then it drops rapidly to very low concentrations [146, 147, 148]. In January, we found almost no SER in the Leydig cells of deer, which corresponds to the lowest level of testosterone in the blood, close to zero. Leydig cells (at least in rodents) have a tremendous regenerative capacity, which has been found by many authors after selective destruction of Leydig cells with ethylene dimethanesulfonate (EDS) [149, 150, 151, 152, 153]. We assume that in roe deer, the inactive Leydig cells full of lipid droplets, remnants of SER and glycogen clusters either regenerate or are removed by macrophages and a new generation of Leydig cells is formed in the spring. Since the duration of apoptosis can range from a few hours to several days [154, 155, 156], it is unusual that we found apoptotic Leydig cells with chromatin margination not only in August but also in January, several months after the rut. Autophagy also occurs at a high rate in Leydig cells [157, 158, 159] and plays a significant role in regulating testosterone production by influencing uptake of cholesterol [158, 160]. In Leydig cells, autophagosomes preferentially sequester organelles important for steroid production such as mitochondria and SER [159, 161, 162]. In the autophagosomes of roe deer Leydig cells, we also found almost exclusively mitochondria and residual SER. Apoptosis plays a vital role in tissue homeostasis and is a vital part of various cellular processes, including normal cell turnover and atrophy [155, 156, 163, 164], and is also involved in seasonal regression of testis spermatogenic activity [165]. In recent years, other cellular mechanisms were found to be involved in the decrease of the activity of the seminiferous epithelium during the non-reproductive period, such as changes in Sertoli cell morphology and function, and autophagy. Autophagy maintains intracellular homeostasis by the process, in which cells degrade and recycle their own proteins and organelles [160, 164, 166, 167, 168, 169]. Both apoptosis and autophagy can occur simultaneously in the same cell [169, 170, 171, 172]; they often occur sequentially with autophagy preceding apoptosis [171]. We also observed both autophagy and apoptosis in the same roe deer Leydig cells. Sertoli and Leydig cells in adults (at least in rodents) are stable cell populations throughout the year that do not normally proliferate [144, 173, 174, 175], even in roe deer, a seasonal breeder [145, 176, 177], though they can regenerate if experimentally eliminated. This could explain that we never observed macrophages in the interstitium of the roe deer testis in the non-breeding season, which remove dead Leydig cells e.g., after EDS [149, 150, 151, 152, 153]. Dead Leydig cells are cleared rapidly, as already "4 and 14 days after EDS, macrophages were the dominant cell in the interstitial space" and 14 days after EDS administration, inclusions from dead Leydig cells in macrophages almost disappeared [150].

The seasonal breeding rodents are used as a model to study structure-function relationships in the testis [e.g., 136, 137, 139, 178, 179]. Klonisch et al. [177] state the roe deer "as an excellent non-rodent model for studying seasonal regulation of testis function with naturally changing photoperiod". We also recommend a roe deer, a ruminant and seasonal breeder, similar to economically important cattle with continuous spermiogenesis, as the best natural model for studying the regulation of spermatogenesis, already proposed by Klonisch et al. [177] without knowing about seasonal circannual changes of nucleolus in their Sertoli cells. Since the nucleolus participates "in the cell proliferation, resting state, differentiation, maturation, ag-

ing and death" [108], the MVN in ruminant Sertoli cells and its annual structural cycle in roe deer is not a cytological rarity or curiosity, but can serve as a simple morphological marker of ruminant Sertoli cell activity. Since membrane vesicles appear in the nucleolus of Sertoli cell precursors of bull calves [77], and MVN in Sertoli cells of bull [82, 89], goat [94, 97, 98], water buffalo [90] and roe deer [73, 88] develops before the onset of spermatogenesis (regardless of whether during puberty or before rut in adult seasonal breeder), we suppose MVN is related to the regulation of spermatogenesis. It has been 140 years since Enrico Sertoli first identified and described cells in the seminiferous tubules of the testes in 1865 that now bear his name. Based on the current PubMed and Scopus databases, approximately 24,000 to 26,000 studies on Sertoli cells have been conducted to date and there are still many reasons and challenges for further research on them. Among other, because of obstacles to the transfer of basic research findings into practical use in reproduction and clinical therapy. More than one-half century has passed since the discovery of MVN, later also known as the vesicular nucleolus, in the bull Sertoli cells and its function remains unknown as well as why it only occurs in ruminants. Further study of ruminant Sertoli cells is also needed to clarify the role of MVN in Sertoli cells and its function for spermatogenesis.

## CONCLUSION

The nucleolus as a vital cell structure, which plays a crucial role in protein synthesis by producing ribosome subunits. Without the nucleolus, cells would not be able to produce the ribosomes necessary for making proteins. Sertoli cells synthesize hundreds of proteins and polypeptides therefore have a very euchromatic nucleus and species-specific prominent nucleolus. The ruminant Sertoli cells' nucleolus consists of membrane vesicles covered with ribosome-like small granules, first described as a multivesicular nuclear body – MNB, later also known as a vesicular nucleolus. Given the large number of round membrane vesicles in it, we suggest a more appropriate name multivesicular nucleolus – MVN. Unlike domestic ruminants, which have continuous spermatogenesis and reproduce continuously throughout the year, and MVN also occurs throughout the year, in the roe deer, a seasonal breeder with seasonal spermatogenesis, the fully developed MVN occurs only before the onset and during spermatogenesis. In January, in regressed testis, the nuclei of Sertoli cells of roe deer lack large membrane vesicles and have a reticular nucleolus. The seasonal MVN development in Sertoli cells of adult roe deer resembles its ontogenetic development in continuously breeding ruminant. The formation, disappearance and lack of membrane vesicles in the nucleolus of adult deer Sertoli cells repeats itself every year, creating a circannual seasonal cycle of the Sertoli nucleolus that precedes the circannual cycle of Leydig cells. The latter mainly consist of the loss of the smooth endoplasmic reticulum after rut, and accumulation and storage of fat droplets and glycogen in inactive Leydig cells. The function of MVB is still unclear. Its structure and deep folds of the Sertoli cell nuclear envelope towards the MVN containing numerous ribosomes suggest its role in ribosome production. Since membranes in the nucleolus of ruminant Sertoli cells appear before spermatogenesis, we believe that MVN play a role in its regulation, at least at its onset. What the exact purpose of the membrane vesicles in Sertoli cells is, and why the MVN is found only in ruminants, still remains a mystery.


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## Conflict of Interest

None of the authors has any conflicts of interest.

## Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

## Ethical Statement

This study did not require any ethical approval, as it is not required if the study material comes from animals slaughtered in an abattoir, from hunted game, or obtained by castration. During the game protection season, animals were shot with exceptions granted by the Ministry of Agriculture and Forestry of the Slovak Republic and the Directorate of East Slovakian Forests.

## Authors' Contributions

M. Z.: Conception and design of the study, specimen collection, electron microscopy, drafting the manuscript, final approval and accountability. K. H.: methodology, processing of specimens, electron microscopy, bibliography search, manuscript revision. J. P.: high-resolution electron microscope photography, manuscript revision. All authors read and approved the published version of the manuscript.

## Generative AI Statement

The authors declare that they did not use any AI generation in the creation of this manuscript.

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The paper is dedicated to the memory of two outstanding researchers in reproduction and in particular Sertoli cells. Lonnie D. Russel (December 11, 1944 – July 11, 2001) was an exceptionally successful researcher with 16,395 citations, passionate about the Sertoli cell. Lennart Nicander (November 4, 1923 – May 8, 1991) was the first to observe membrane vacuoles in the nucleolus of a bull Sertoli cell.