

This publication will give students and engineers of animal production understandable and clear scientific insight in to authors theoretical knowledge, as well as laboratory work in part of animal biotechnology concerning ultrastructure of rabbit gametes and stem cells. The publication should be a supplement to the literature in field of biology, reproduction and embryo technology.

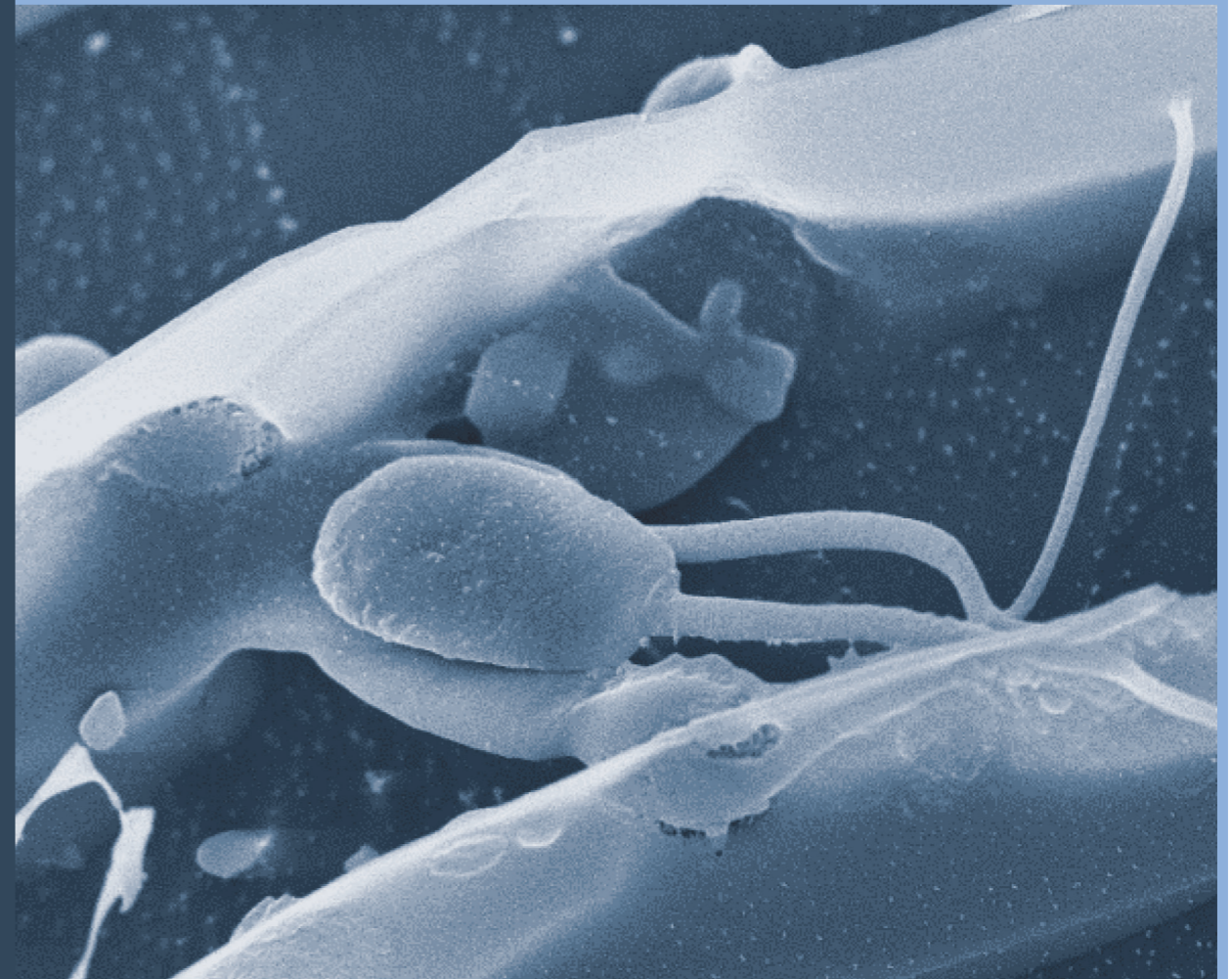
Presented monograph is written in highly skilled and vocational manner, achieving to point out great importance of Stem cells research in modern reproductive biotechnology. Significance of Stem cells are in fact that they are defined as undifferentiated cells that are capable of self-renewal and differentiating into many different cell types, which can produce at least one type of highly differentiated descendant. This characteristic of Stem cells are giving us opportunity to use them in many ways, such as: exploration of genetic and biochemical processes in animal reproduction, curing genetical and hereditary diseases, developing bioreactors for „designer“ tissues and organ engineering, conservation of indangered species, and many more scientific and practical use that this great biotechnological find has put in front of us. In this publication reader can see part of authors years-long experience in embionic cells research.

The authors used great reproduction potential of rabbit that enables easier and faster understanding of a number of biological patterns related to physiological and biochemical processes of Stem cells. Short generation interval and opportunity to collect large number of gametes and embryos gave authors chance to explore ultrastructure of gametes and conclude clarification of their functions that is essential for the successful management of laboratory and clinical practices in animal reproduction. This includes also knowledge of the functions and structure responsible for the maturation and production of gametes, understanding of the developmental patterns of gametes and early embryos.

Chrenek et al.

Ultrastructure of Rabbit Gametes and Stem Cells

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2017



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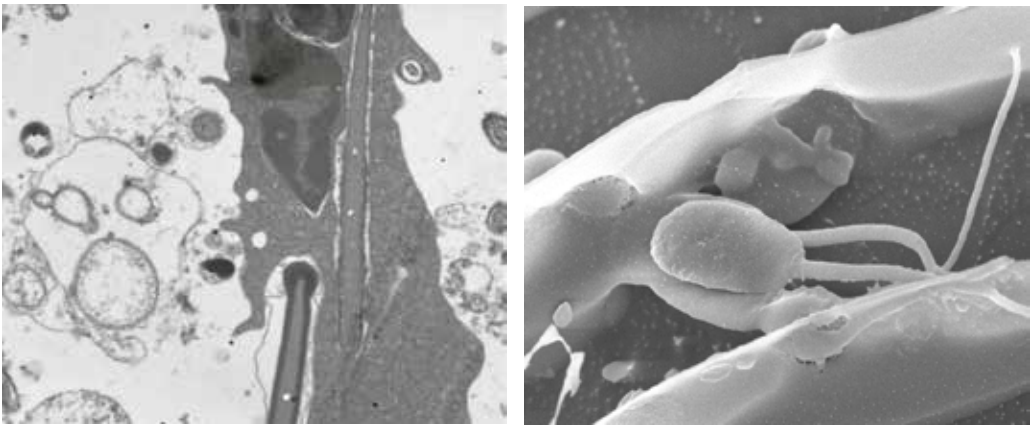
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Introduction

Characterization of reproductive cells (gametes) and clarification of their functions is essential for the successful management of laboratory and clinical practices in animal reproduction. For proper understanding of the developmental patterns of gametes and embryos, knowledge of the structure and functions which are responsible for the production and maturation of gametes, but also for fertilization and early embryo development is necessary. This publication will help students and university teachers to improve their spatial imagination at studying dynamic processes of rabbit reproductive and stem cell development. Rabbit as a proper biological model, enables easier and faster understanding of a number of biological patterns related to that topic.

The authors at the processing of this publication took into account requirements to the student in terms of theoretical knowledge as well as laboratory experience. For these reasons, the authors included into the publication various biological material containing oocytes, embryos, two types of stem cells (mesenchymal and amniotic) as well as spermiophagic cells of rabbit. At the processing and analysis the authors used several basic as well as modern and more effective methodical approaches and equipment. Substantial part of the monograph is formed by photographs from transmission electron microscopy, and also representative images from scanning electron microscope. Electronograms of normal and abnormal spermatozoa were evaluated following classical analysis by transmission electron microscope. Embryos and stem cells were also imaged using electron microscope in a transmission mode.

The publication should serve as a supplement to the textbooks focused on the problems of biology, reproduction and embryo technology. The monograph must not be taken as a substitute for student's textbooks in embryology and andrology, in the study of oogenesis, spermatogenesis, fertilization, early embryonic development, but as a complement to expand the topic being studied. It is intended for students of biotechnological specializations, natural sciences, as well as for students of human and veterinary medicine.

The pictures used in the monograph are original and were obtained at the workplace Research Institute of Animal Production, NAFC Nitra and workplace VC AGROBIOTECH SPU Nitra. Cryoscanned images were acquired in cooperation with CEPOMA STU Bratislava.

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1. Characterization of rabbit reproductive cells (oocyte, spermatozoa, embryo)

Characterization of an oocyte and embryo

The aim of the study of different developmental stages of the egg from the oocyte to the blastocyst stage embryo is their morphological characterization. Embryos, which are deviated from normal development, must be eliminated thereby increasing the success rate of genetic manipulation and embryo transfer.

a) Oocyte

Immobile oocyte, surrounded by the follicular cell layer (Fig. 1.1) designed as the *corona radiata* (Fig. 1.2), by the action of LH hormone leaves the ovary. This layer during the ova staying in the oviduct is losing (Fig. 1.3), both in the process of fertilization and also due to the friction with the wall of the oviduct. These cells are involved in the oocyte nutrition and the mediation of some signals coming from the oocyte before ovulation. Under a layer of follicular cells there are cumulus cells and elastic membrane - *zona pellucida*. *Zona pellucida* has the ability to last until the blastocyst stage and plays an important role in fertilization, i.e. in relation to the binding of sperm to the egg (sperm-egg binding). Under the *zona pellucida* the ooplasm membrane is laying, which delineates the cytoplasm. In the ooplasm, there are the cell

organelles specific for the animal cells, such as the nucleus with the haploid number of chromosome after second meiotic division.

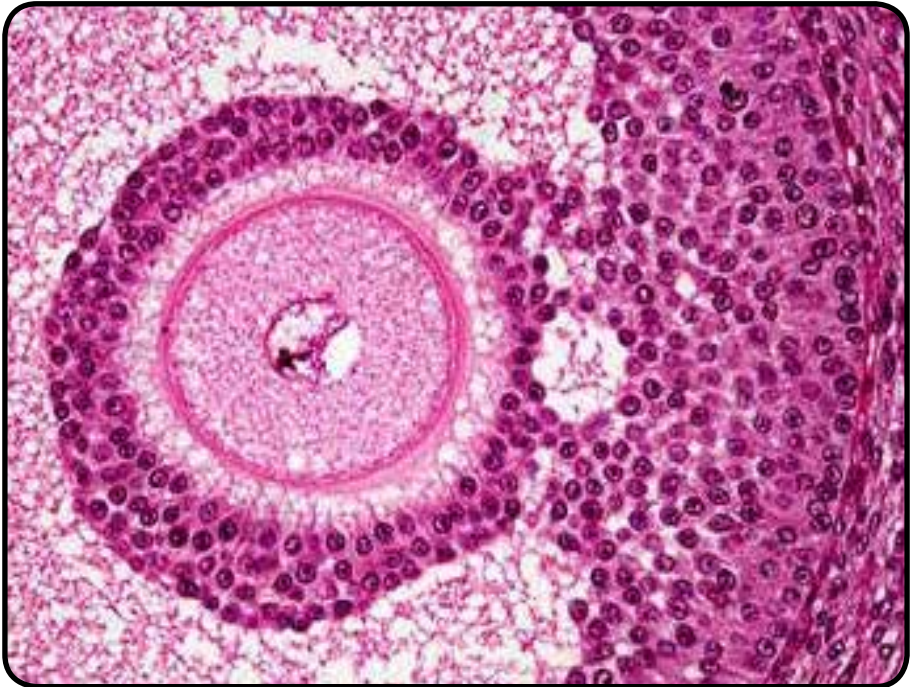


Figure 1.1 The oocyte before ovulation <http://micro.magnet.fsu.edu/primer/anatomy/brightfieldgallery/images/mammaliangraafianfollicle40xsmall.jpg>

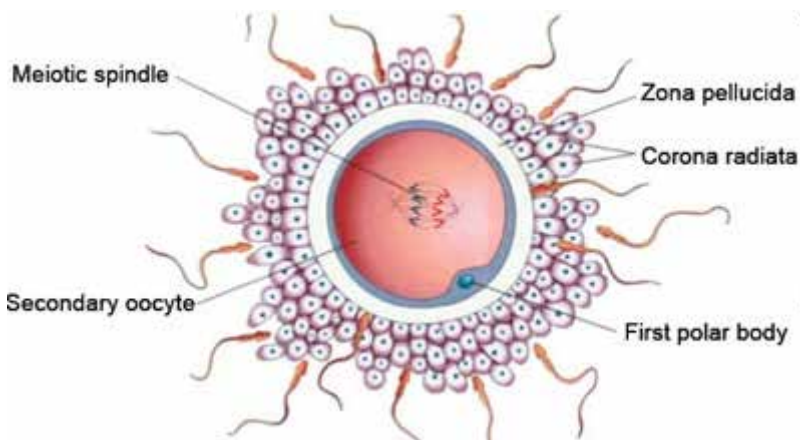


Figure 1.2 Scheme of the oocyte with cumulus cells closely after ovulation http://www.thekeytoislam.com/en/assets/images/g_fertilization_1.jpg



Figure 1.3 Ovulated oocyte without cumulus cells

Morphological characteristics of cumulus cells together with the morphology of the oocyte cytoplasm are important for the oocyte evaluation. Říha et al. (1999) defined five morphological grades of oocytes:

- 1) oocyte with complete *corona radiata* consisted of more than 6 layers of cumulus cells and moderately dark, homogenous and finely granulated ooplasm;
- 2) oocyte with incomplete but compact *corona radiata* consisted of 3-5 layers of cumulus cells with moderately dark and homogenous ooplasm;
- 3) oocyte surrounded by 1-2 layers of cumulus cells with moderately dark or dark ooplasm containing several necrotic foci;
- 4) oocyte with only residual sporadic cumulus cells with dark and non-homogenous partially fragmented ooplasm;
- 5) oocyte without cumulus cells with fragmented ooplasm.

b) Zygote

Fertilization leads to the fusion (syngamy) of the paternal pronucleus, formed from the sperm head, and maternal pronucleus, formed by the nucleus splitting at the second meiotic division. In this case we are talking about the one-cell stage egg - zygote (Fig. 1.4). This stage of development, which only takes 3-6 hours, is characterized

by compact *zona pellucida*, two or three polar bodies in the perivitelline space of the zygote, the cytoplasm with two pronuclei and, depending on the animal species, the higher or lower occurrence of lipid inclusions (Chrenek et al., 1999).



Figure 1.4 Fertilized rabbit egg at the stage of two pronuclei

c) Embryo

The first division of the zygote (in rabbit - 24 hours after fertilization) results in the 2-cell embryo (or embryo at the stage of two blastomeres, Fig. 1.5). After subsequent divisions 4-cell, 8-cell, 16-cell stage embryos occur (Tab. 1.1).



Figure 1.5 Rabbit embryo at the 2-cell (2-blastomere) stage of preimplantation development

Table 1.1 Chronology of embryo division in several animal species

Species	2-cell	4-cell	8-16-cell	Morula	Blastocyst
Cow	36 hour	72 hour	96 hour	6-7 day	8-9 day
Sheep/goat	36 hour	42 hour	72 hour	4-5 day	6-7 day
Mare	24 hour	30-36 hour	96 hour	5 day	6 day
Sow	25 hour	50 hour	96 hour	5 day	6 day
Rabbit	24 hour	35 hour	48 hour	3 day	4 day
Mouse	22 hour	32 hour	40 hour	3 day	4 day

All these developmental stages are, under optimal conditions, characterized by compact and entire *zona pellucida*, regularly dividing and homogeneous blastomeres (Fig. 1.6).



Figure 1.6 Rabbit preimplantation embryo at the 16-cell stage

Beginning from the 16-blastomere stage the embryo is called „morula“. The subsequent development stage, when the blastocoel cavity begins to form and the cells begin to differentiate into trophoblast cells and the cells of the inner cell mass, called a compact morula (Pivko et al., 1988).

The next developmental stage is the blastocyst, when differentiation of the outer layer of trophoblast from more compact inner cell mass, also referred to as embryonic stem cells, is already visible. The expanded blastocyst stage is a next developmental stage, when enlargement of the outer diameter of the embryo is clear and the narrowing *zona pellucida* cracks. When the blastocyst leaves the *zona pellucida*, we are talking about free (hatched) blastocyst (Fig. 1.7).



Figure 1.7 Rabbit embryo at the hatched blastocyst stage

There is a classification of different developmental stages using qualitative criteria according to Pivko et al. (1988):

- 1) excellent** – an ideal embryo, round-shaped, symmetrical, with cells of even size, similar colour with compact and homogenous cytoplasm in each blastomere;
- 2) good** – occurrence of several fragments of blastomeres in the perivitelline space, uneven shape and colour, vesicle incidence;
- 3) fair** – higher occurrence of fragmented blastomeres, several degenerated cells in the perivitelline space, presence of vesicles;
- 4) insufficient** – visible disorders in the development, significant blastomere fragmentation, degenerated and differently-sized cells .

Characterization of spermatozoa

Spermatozoa is a functional male gamete with a haploid number of chromosomes, which is created in male reproductive organs and is able to fertilize female egg. It consists of a sperm head, midpiece and tail (Fig. 1.7). Sperm are the most variable animal cells, and there is very large phenotypic variability among species. The whole sperm is covered with a plasma membrane. The sperm head consists of the nucleus and the acrosome. The nucleus contains a male DNA, and the

acrosome contains important enzymes which are essential for the fertilization of the egg. The midpiece segment connects the sperm head and flagellum (tail). It contains various number of mitochondria arranged spirally around the frontal part of the sperm tail, that supply energy to the sperm tail (Ljiljak et al., 2012).

Sperm are the final product of spermatogenesis. They are highly specialized final cells, whose function is to fertilize female gamete. They are transported to the epididymis, where they are maturing. Sperm are labile cells. Their plasma membrane has an inherent ability to be destabilized under the influence of different stimuli from neighboring membranes during the acrosomal reaction (Rodriguez-Martínez et al., 2001).

The release of acrosomal content is an exocytosis process, called acrosomal reaction, which occurs only in the vicinity of the oocyte. The absence of acrosome indicates that sperm must not be fully functional and, therefore, cannot fertilize an egg. However, before the sperm are able to undergo acrosome reaction, it must pass a series of maturation steps involving thoroughly regulated intracellular signaling and phosphorylation of proteins, which is called "sperm capacitation" and takes place in the female reproductive system prior to the acrosome reaction and fertilization of the egg (Breitbart et al., 2005; Ramalho-Santos et al., 2007).

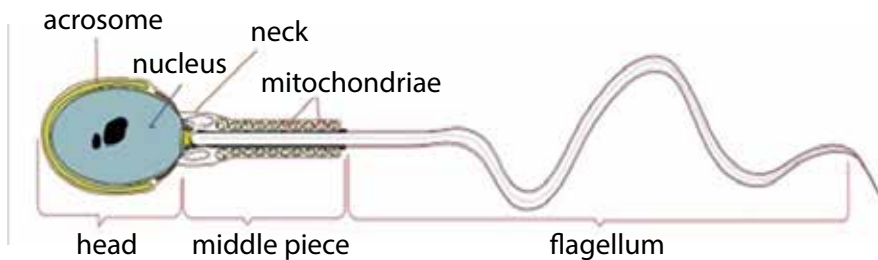


Figure 1.7 Morphology of spermatozoa

(http://www.podpora-plodnosti.cz/JAK-FUNGUJI-SPERMIE-a5_65.htm)

Flagellum (tail) of the sperm is critical for its motility. It consists of the axoneme containing typical microtubular arrangement of the nine double pairs of peripheral microtubules arranged in a circle around a central pair (Fig. 1.8). Another protein structure, formed by the outer dense fibres and the fibre jacket, surrounds the axoneme and provides the power and the strength of the flagellum (Ramalho-Santos et al., 2007).

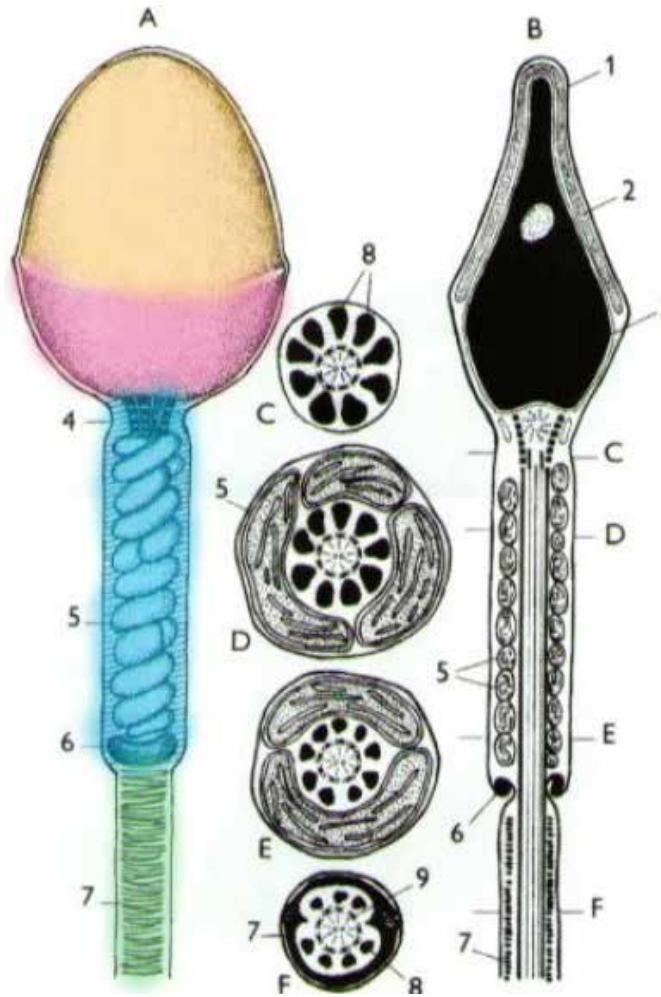


Figure 1.8 The scheme of a spermatozoa drawn according to electronograms

A – a side view of the plasma membrane, B – longitudinal section,
 C-F – cross-sections through the flagellum at different levels,
 1 – plasma membrane, 2 – acrosome, 3 – the nucleus with the vacuole,
 4 – the neck with two centrioles, 5 – mitochondria, 6 – Jensen ´ ring,
 9 – (7,8) microtubules of the flagellar axoneme
 (Pivko et al., 2013)

Knowledge of the sperm structure is dated from the 17th century, when Anton van Leeuwenhoek first described the existence of these cells in the seminal fluid of animals and humans. He defined mor-

phological abnormalities of the sperm head. Intensive research during the 18th and 19th centuries precisely determined the origin and the essential function of sperm in fertilization of the female gametes. The implementation of advanced morphological, biochemical and molecular techniques into reproductive biology has led during the 20th century to the characterization of a variety of sperm abnormalities (Fig. 1.9) (Chemeset and Rawe, 2003).

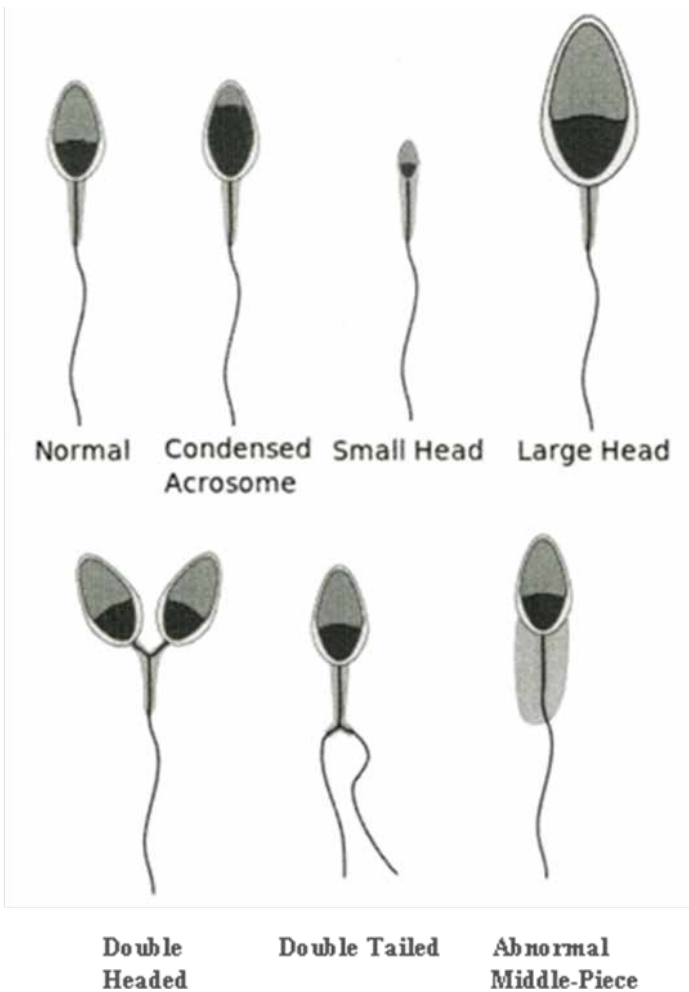
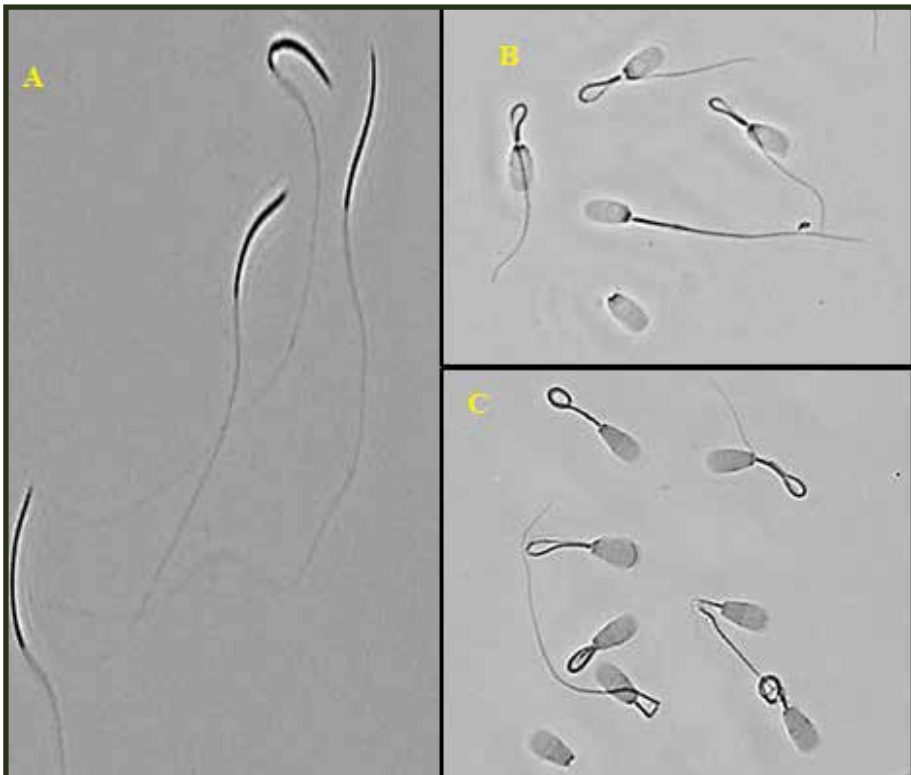


Figure 1.8 Sperm abnormalities
http://en.wikipedia.org/wiki/Semen_analysis

Interspecies differences

Ejaculates of different animal species vary in the sperm concentration, ejaculate volume, composition of the seminal plasma and sperm shape (Fig. 1.10). A common feature, however, is the mobility and fertilizing ability (Castellini et al., 2006; Mocé and Vicente, 2009). Bull ejaculate may have volume of 2 - 8.5 ml (depending on breed) at a concentration of 800×10^6 sperm per ml. Sperm motility in ejaculate ranges from 55% to 75% containing 55-85 % of live and 15-45 % of dead spermatozoa. Ram ejaculate is characterized by a small volume (0.5-2 ml) but high concentration of spermatozoa ($2000-5000 \times 10^6$ /ml), in contrast to the semen of boars and stallions. The volume of the semen of boars is in range of 150-200 ml and in stallions - 20-300 ml, which is incomparably more than in ram, bull or rabbit. Sperm concentration in boar ejaculate is only $25-350 \times 10^6$ /ml, and in a stallion ejaculate $30-800 \times 10^6$ /ml (Gordon, 2004; Chenoweth and Lorton, 2012; Rahman et al., 2014).



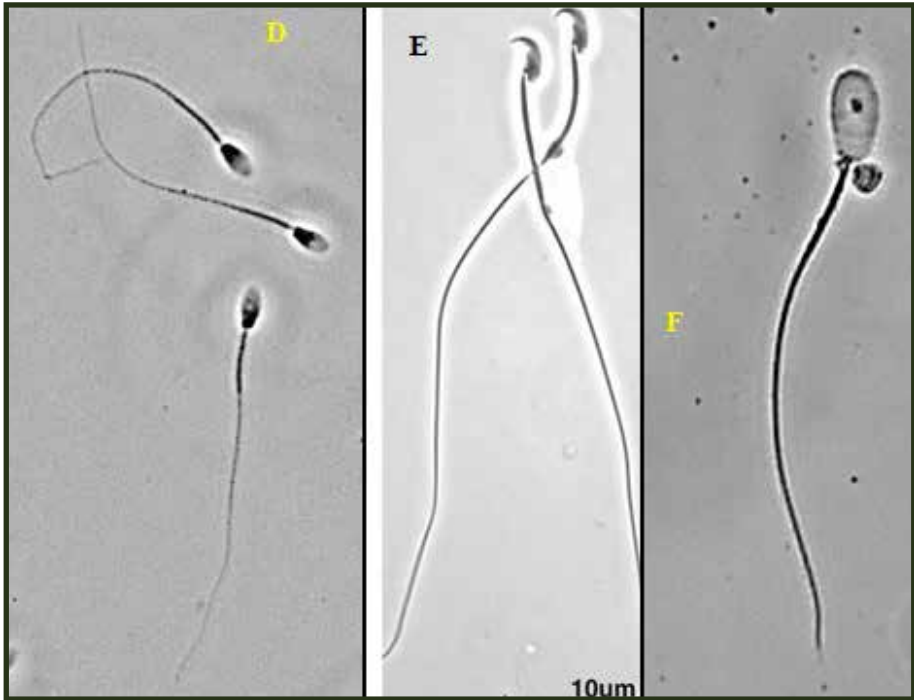


Figure 1.10 Spermatozoa of different animal species: A - rooster, B - boar, C - bull, D - stallion, E - mouse, F - rabbit (http://www.ansci.wisc.edu/jjp1/ansci_repro/lab/lab9/sperm_images/sperm_images.html)

Characterization of rabbit ejaculate

Rabbit semen, in contrast to other domestic animals, is characterized by low volume and low sperm concentration (0.4 – 0.5 ml; 150 - 500 x 10⁶ sperm/ml). These parameters depend on the breed and lineage. Glycoprotein in rabbit seminal plasma, which comes from the epididymis, reversibly inhibits sperm capacitation process. Rabbit seminal plasma contains droplets and vesicles of similar size as the spermatozoa. The function of these droplets and vesicles is unknown, it may have different role in the modulation of different functions of sperm (motility, capacitation, acrosomal reaction). These droplets are very rich in cholesterol and serve as donors of sterols for protection against cold shock in spermatozoa and premature acrosome reaction (Moce and Vicente, 2009).

2. Characterization of rabbit stem cells

Cholesterol modulates the membrane fluidity by interaction with the phospholipidic fatty acyl chains, and the ratio of cholesterol and phospholipids is an important determinant of the membrane fluidity. Unlike the sperm of other species of domestic farm animals, the rabbit sperm plasma membrane contains a high ratio of cholesterol-to-phospholipids and low ratio of polyunsaturated and saturated fatty acids in phospholipids, which is similar to human sperm. These features increase the secondary membrane fluidity, thus sperm is able to withstand greater environmental stressors. For this reason rabbit sperm is characterized by higher resistance to cold shock (Castellini et al., 2006; Mocé and Vicente, 2009).

Every mammalian organism is formed by three basic cell types: germ cells, somatic cells and stem cells.

Germ cells give rise to gametes, eggs and spermatozoa.

Somatic cells represent the largest group of cells. These are all the cells of the body, except germline cells, i.e. cells forming bones, blood and organs of mammals. Each somatic cell has a nucleus, excepting nucleus-free cells like erythrocytes.

Stem cells are undifferentiated cells, which have a capacity to differentiate into cell types that are not part of the tissue from which these stem cells are derived. This property is designated as plasticity of a stem cell. Stem cells are also considered as cells with "slow" cell cycle, but with high clonogenicity. Generally, they represent only a small percentage of all cells that form a certain organ.

There are three criteria that cells must meet in order to be designated for stem cells:

1. They must be able of self-renewal by means of symmetric or asymmetric division, whereby the stem cell population is maintained. Daughter cells resulting from the symmetrical division fully retain the characteristics of stem cells, while at the asymmetric division only one the resulting cell maintains the characteristics of a stem cell, but the other one is differentiated.
2. Single cell must be capable of multilineage differentiation into many cell types.
3. The cells must be capable of in vivo re-population and functional reconstitution of the tissue.

Stem cells of adult organisms – somatic stem cells (SSC) or adult stem cells (ASC) are different type cells located in the tissues and organs. It were found in the blood, bone marrow, skeletal muscle, cornea and iris of the eye, in the dental pulp, brain, spinal cord, in the blood vessels, liver, skin, gastrointestinal tract and pancreas. The main function of adult stem cells is permanent renewal and replace of worn adult specialized cells. They are present in all organs, where they serve as cellular reserves.

Historically, the “adult” stem cells were first identified in the hematopoietic system on various animal models. At that time it was assumed that the main biological function of stem cells is the protection from radiation, the formation of specialized germ cells of different lines and self-renewal. The name “adult” is used to differentiate multipotent cells from embryonic stem cells and embryonic germ stem cells.

It is assumed that adult stem cells in the majority of differentiated undamaged tissues are unipotent, and under normal conditions only one cell type is originated from them. However, in case when tissue is damaged and a replacement of more cell types is required, pluripotent stem cells can be activated in order to repair damage (Slack, 2000).

Somatic stem cells are pluripotent (Avasthi et al., 2008). With rare exceptions, all somatic stem cells of adult organism can give rise to cells of different types. Usually at the differentiation, somatic stem cells follow the principle of co-localization of SSC with an effector, i.e. SSC differentiate into the cells of an effector organ or tissue, where they are located. The mentioned exceptions are hematopoietic stem cells (HSC), which can differentiate into any type of blood

cells, but normally are located almost exclusively in the bone marrow, which is not an effector organ of the circulatory or immune system. However, the principle of co-localization of stem cells and the effector function is fulfilled in the early embryogenesis - at this stage HSC are located in the lumen of the aorta. The above mentioned, as well as the fact, that the bone marrow is also represented by other cells, suggests that the bone marrow could not be only hematopoietic tissue, but also specific organ, the main function of which is to maintain homeostasis of the circulatory and immune system, bone, and possibly of other systems.

Somatic stem cells are very few and they are scattered along the tissue. In contrast to embryonic stem cells, whose origin is clear already from their definition, little is known about the origin of the SSC in adult tissues. Similarly, molecular basis of SSC differentiation into adult cells (except hematopoiesis) has not been investigated. On the basis of certain data and by analogy with the hematopoietic stem cells, it is assumed that the SSC are governed by the principle of hierarchical stepper maturation which is strictly directed and irreversible. The least differentiated, primitive SSC are mostly quiescent. The result of their division are more differentiated transitional cells called progenitor (germ) cells. These cells form rapidly proliferated population, whose pluripotency is significantly reduced, and the cells specialize more towards definitively adult cells. Stem cells give rise to differentiated cells over several generations of transitional cells, each generation of which is more and more differentiated.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) were, for the first time, identified and isolated from the bone marrow (BM) more than 40 years ago, when researchers had shown that bone marrow contains hematopoietic stem cells and stromal cells, which are characterized by high plasticity and adherence. Nowadays, these cells are called mesenchymal stem cells. The unique properties of MSCs, such as an easy amplification and isolation from bone marrow, immune tolerance and multilinear potential, supported the study for curable treatment of heart diseases. Later it was shown that the MSC can differentiate into

a variety of cell types: osteoblasts, chondroblasts, myoblasts, fibroblasts, adipocytes and stromal cells.

MSCs are assigned to the stem cells, which are unable of forming a blood cell lineage, i.e. to the non-hematopoietic stem cells. Using flow cytometry it was found, that MSCs express the following surface markers: CD29, CD44, CD73, CD90, CD105, CD106. On the contrary, markers which we do not find on MSCs are CD31, CD33, CD34 or CD45. These markers are typical for hematopoietic stem cells (Al-Nbaheen et al., 2013).

Amniotic stem cells

Amniotic fluid (*liquor amnii*) contains heterogeneous cell population, whose composition depends on gestational age or pregnancy stage. These cells originate from the foetal tissue and amnion (Fig. 2.1). During pregnancy, a certain proportion of these cells lose viability.

Amniotic fluid (AF) fills the amniotic cavity and prevents the adhesion of the embryo to the amnion, protects the embryo or fetus against mechanical impacts and allows movement of the fetus. Part of AF is produced by amniotic membrane, but a substantial part comes from the mother's blood. Fetus consumes AF and replaces it with fetal urine. Fetal urine is mostly a water with waste products of metabolism which are removed via placenta (Sadler and Langman, 2006).

Amniotic cavity is formed by a space between the embryonal ectoderm and mesoderm in the second week of human foetal development (Beck et al., 1995).

The number and proportion of cells of the amniotic fluid varies over the course of pregnancy. Their main source is the skin, at the beginning embryonic, from the surface layer (*peridermis*) of which the cells peel off into the amniotic fluid. Later the skin acquires definitive character of an *epidermis*, and keratinized cells peel off into the amniotic fluid. The cells of *epidermis* are the main component of AF cells. Another source of cells present in the amniotic fluid is an amniotic ectoderm. A smaller part gets into the amniotic fluid from the urogenital outlets, in females mainly from the vagina; some portion comes from the gut tube.

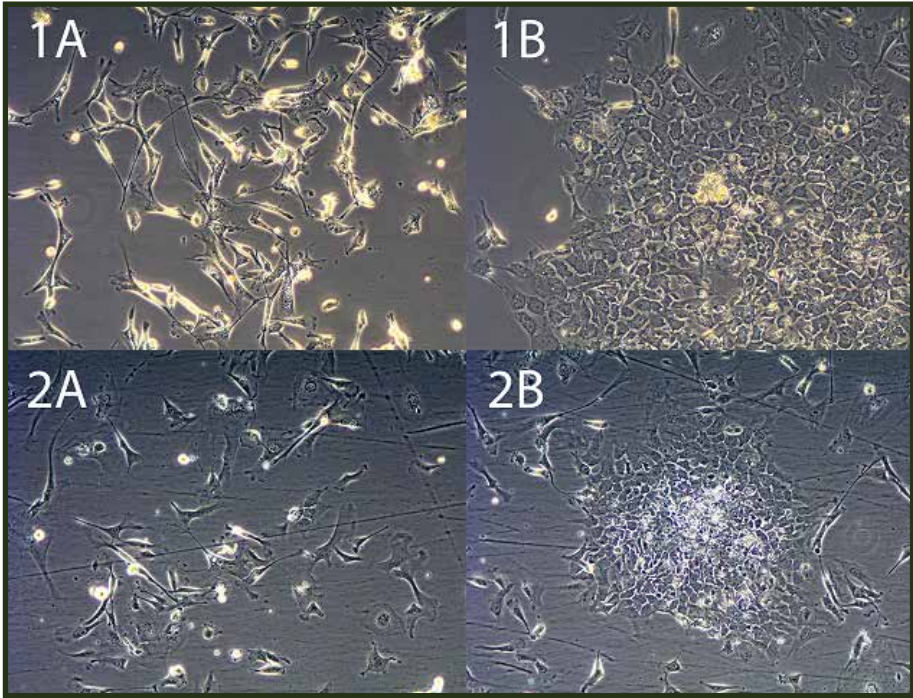


Figure 2.1 Rabbit amniotic stem cells

3. Characterization of sperm phagocytosing rabbit cells

Phagocytosis is regarded as a mechanism that is similar to the absorption of food by amoebas. Such ability to absorb surrounding particles is specific to a number of cell types. In higher organisms, there is a cell population, which is able to ensure the immunity of the organism by a mechanism of phagocytosis. Cells of this type are called professional phagocytes. These include eosinophilic and neutrophilic granulocytes, monocytes, their tissue form - macrophages, which are the basic cells of a non-specific immunity and the central cells of an inflammatory response. Atypical sort of phagocytic cells are dendritic cells, whose primary function is the representation and processing of antigens (Horejsi and Bartůňková, 2009). Phagocytic cells have different characteristics, in particular: granulocytes are important for the anti-infectious defense, mainly against extracellular bacteria; macrophages phagocytose the remnants of own cells derived from apoptosis and, at the same time, significantly participate in the defense against some intracellular parasites. Granulocytes have ability to perform their effector functions immediately, but the macrophages become fully functional only after activation by 23 signals, which will be supplied from T-lymphocytes in form of cytokinins (interferon- γ , TNF). Neutrophils are short-living cells, macrophages and dendritic cells live quite a long time and have the ability to transform in different ways, or pass through the various stages of activation (Horejsi and Bartůňková, 2009).

Phagocytosis is the cell process of absorbing solid particles from the outside, which is mediated by actin. Specialized cells, such as white blood cells or macrophages, absorb by means of endocytosis bacteria, viruses, or foreign and damaged cells from their surroundings.

Absorbed particles are usually larger than about 0.75 μm in diameter. Phagocytosis is often very specific, it can be conditioned by binding a specific antibody to the bacterial surface (Pollard et al., 2008).

Development of the mononuclear phagocyte system (Fig. 3.1) begins in the bone marrow from a haematopoietic stem cell (HSC), which is a common precursor of all blood cells. In the next step HSC differentiates into the normal myeloid progenitors (CMP), which already has limited differentiation potential. This leads to the formation of granulocyte-macrophage precursor (GMP), and subsequently dendritic macrophage progenitor (MDP). MDP is further differentiated into two branches: immature elements of the dendritic series and the monoblasts. Monoblasts give a rise to pro-monocytes and monocytes. Monocytes leave the bone marrow and reach the peripheral blood, where remain for several days and then entered the tissues to complement the tissue macrophages (Chow et al., 2011).

Only a special group of cells, called professional phagocytic cells, use the mechanism of phagocytosis for a defense and for disposal of unwanted microorganisms and particles, which invade the organism. Professional phagocytes have the ability to find, absorb and kill the microorganisms, damaged and aging cells and other foreign material of 0.5 μm or more in size (Krejsek and Kopecky, 2004).

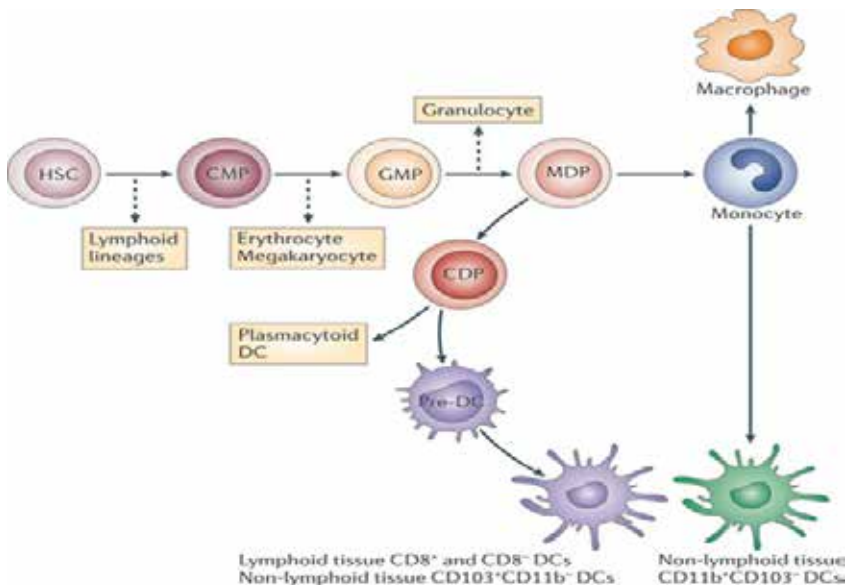


Figure 3.1 Development of mononuclear phagocytosis system (Chow et al., 2011)

The course of phagocytosis

Phagocyte, at the beginning, contacts foreign particles by a small area of their surface. Foreign particle is contacted via various receptors located on the surface of a phagocyte. Macrophages are attracted to the site of inflammation not only by bacterial products or chemotactic agents of the immune system, but also by the substances, which are released from damaged cells and extracellular matrix (Buc, 2009). Foreign particle is gradually surrounded by phagocytic pseudopodia. The particles are completely enveloped by the surface membrane of a phagocyte and are subsequently enclosed into the newly formed vacuole called phagosome. The formation of the phagosome is dependent on the intracellular rearrangement of contractile proteins. These processes are controlled and initiated by signals provided by the receptors on the surface of phagocytes (Horejsi and Bartůňková, 2009).

Characterization of macrophages

The macrophages are present throughout the animal organism. They are found in lymphoid as well as in non-lymphoid organs. They are also present in the liver (Kupffer cells), lungs (alveolar macrophages) and are the part of the nervous system (microglial cells) and the reproductive organs (Taylor et al., 2005). Macrophages are usually the cells with a one nucleus with the size of 16-22 μm (Rovenský et al., 2006). Macrophages make up about 5% of the entire population of leukocytes. They have a high content of the cytoplasm, where the round-shaped or kidney-shaped nucleus is located. The macrophages derived from stem cells of the bone marrow called monoblasts. Monoblasts, under the influence of CSF (colony-stimulating factor) differentiate into promonocytes. Monoblasty sú pod vplyvom rastových faktorov CSF (colony-stimulating factor, angl.) diferencované na promonocyty.

Promonocytes within 6 days are differentiated into the monocytes, which then pass into the blood stream. Monocytes circulate 3 days and subsequently settled in tissues, where they are differentiated into macrophages, some of which, in contrast to neutrophils, can divide. The macrophages are long-living cells, which are renewed at a rate of approximately 1% per day, until they are activated by tissue injury or inflammation process (Buc, 2009).

We distinguish between two basic groups of macrophages: resident - normal macrophages and inflammatory macrophages or exudative. The name "activated macrophages" is typical for macrophages with specifically enhanced functional activity. Resident or normal macrophages are immunologically relatively „quiet“. They are characterized by low consumption of oxygen, small number of the HLA antigens of class II on the surface, and low or no cytokine secretion. However, they have the phagocytic and the chemotactic ability and can maintain certain proliferative capacity (Hulin et al., 2005).

Macrophages, in contrast to neutrophils, can be applied mainly at subacute or chronic inflammation. The material of a foreign origin, which persists in a given deposit for a long period, can surround a number of macrophages, so that at histological examination they look like epithelium and, therefore, called "the epithelial cells". Epithelial cells are characterized by a high content of cytoplasm and numerous amounts of lysosomes and endoplasmic reticulum. These cells, in an attempt to surround and absorb very large particles, can join together to form multinucleated giant cells (Buc, 2009).

Macrophages are characterized by an important role both in specific and non-specific defense mechanisms. They synthesize a number of cytokines, are initiators of a specific immune response, like cells presenting an antigen, they participate in the phagocytosis and are also the component of a complement. Another important role of macrophages is the removal of dead apoptotic cells (Buc, 2009).

Macrophages in male reproductive system

Leukocytes and macrophages have been found in testes of most mammals and are very likely to occur in all mammals. Monocytes are getting into the gonads during fetal life and are considered as a major source of testicular macrophages. Their function and the number of macrophages are significantly affected by the surrounding where they currently occur. According to current knowledge, macrophages can undergo proliferation within the testis during sexual development, but in some cases also in the adulthood (Frungeri et al., 2002). Histological analyses of the male genital system suggest that the macrophages mainly originate from the interstitial cells of testis and epididymis (Tremellen et al., 2010). From the biological point of view,

the meaning of these cells for the function of the testicles is unknown, although it is assumed that their function is immunological. It is possible to observe a phagocytic ability of macrophages following application of a Leydig cell-specific cytotoxin called etyléndimetánsulfón (EDS). The macrophages are characterized by a considerable ability to secrete substances which are biologically active. At present, more than 100 secretory products of macrophages are identified (Frungeri et al., 2002). In other studies the macrophages contribute to the regulation of reproductive function in males, in particular by regulating steroidogenesis, activity of Sertoli cells, and the survival of germinal cells. The current studies emphasize the important function of macrophages in the immunological surveillance in case of sperm quality (Pace et al., 2005).

Macrophages have phagocytic function, are characterized by the production of cytokines, have the ability to inhibit or kill pathogens and tumour cells. According to Sawamoto et al. (2003) macrophages are divided into three basic types:

- exudative macrophages
- resident macrophages
- cells differentiated into dendritic cells

These cells may differ in morphology, tissue distribution, ontogeny and in different functional properties. The exact difference in the number of spermatozoa produced by the testes and the number of cells present in the ejaculate is a sign of resorption and/or sperm phagocytosis by macrophages. Solis et al. (2003) distinguishes in the ejaculate the following three types of phagocytic cells:

- small polymorph leukocytes in size of 10 - 12 μm
- monocytes in size of 10 - 12 μm
- macrophages of size of 30 – 40 μm with the ability to absorb a greater amount of sperm heads

According to Solis et al. (2003) sperm phagocytosing cells get rid of abnormal and degenerated sperm, thereby improving the quality characteristics of the ejaculate. The amount of phagocytic cells in the ejaculate correlates with increased number of sperm with the denaturation of DNA. In inactive macrophages, large membrane folds and globular structure were found at the observation by scanning electron microscopy (SEM).

Active macrophages had the reduced volume and numerous vesicles or granules formed on their surface. The occurrence of inactive

forms has been recorded rarely. A few minutes after mixing of the various fractions of the ejaculate, such level of phagocytosis was achieved, that a portion of the sperm was covered with macrophages, and in some cases first covered were sperm heads, in other cases - the main part of the sperm tail and very rarely connecting segment of the tail (Blanco et al., 1992).

Detection of macrophages

Macrophages and neutrophils represent together up to 95% of all leukocytes that are occurred in the ejaculate. They are characterized by the ability to impair sperm through the formation of ROS, induction of apoptosis, or protease formation. Currently used cytological techniques dealing with quantifying the activity of leukocytes in the semen (peroxidase, CD45) are insufficient, because they provide only the numbers of leukocytes, but not their activity. Elastase of the seminal plasma can indicate effectively an activity of neutrophils, but you cannot use it to determine the activity of macrophages.

Neopterin, a molecule secreted from active macrophages, is considered as an effective marker of macrophage activity, and its amount is determined by ELISA. The incidence of macrophages in various tissues can be detected by means of proper dyes (Tremellen et al., 2010). Solis et al. (2003) in his study used for labelling phagocytic cells in the man ejaculate the stain called Neutral Red, and this stain is able to mark lysosomes in the form of dots in various cell types. Testicular macrophages contain lysosomes of different shapes, what is most likely related to the phase of phagocytosis (Hutson, 2006). These lysosomes in macrophages after staining aggregate into one larger unit of red color, which can then be detected by the microscope (Herbomel et al., 2001). In several studies, the presence of macrophages in the tissues was revealed using acetylated LDL (Goldstein et al., 1978; Parthasarathy et al., 1986).

In case of acetylation of LDL (low-density lipoprotein) complex, a newly formed LDL complex cannot bind to the LDL receptors, and instead is taken up by macrophages i.e. sperm phagocytosing cells. The fluorescent signal, generated by AlexaFluor AcLDL reagent, is able to identify sperm phagocytosing and endothelial cells in a mixed cell population (Solis et al., 2003).

4. Assessment of gametes and stem cells quality

Assessment of oocyte and embryo quality

Currently, great attention is devoted mainly to the use of latest molecular biology techniques in the evaluation and selection of viable oocytes and embryos. Many of these techniques are still under development and their application requires high-quality instrumentation and trained personnel. These are factors that significantly affect the choice of assessment methods in given laboratory conditions.

Generally, at assessing the quality of oocytes and embryos two basic approaches are applied, in particular the use of classic or current methods of evaluation of oocytes and embryos. Both these groups involve invasive and non-invasive techniques.

Classic methods

The group of classic non-invasive methods include visual inspection of the embryos by light and polarized light microscopy (Caamano et al., 2010; Coticchio et al., 2010), which is most often used for the selection of embryos based on the morphology and developmental dynamics. For the evaluation and classification of embryos the following parameters are used: assessment of the pronucleus, structure and location of the polar body, the appearance of the cytoplasm and the *zona pellucida*, embryo cleavage, number of blastomeres on the day of culture, size, shape, symmetry and fragmentation of blastomeres, and the occurrence of more than one nucleus in the blastomere.

Another group of classical methods includes staining techniques used for the differentiation of living and dead cells. The most commonly used staining techniques are with Trypan blue, Neutral red, Brilliant Blue, DAPI (4,6-diamidino-2-phenylindole), propidium iodide (PI), Hoechst 2,5'-bi-1H-benzimidazole, 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl), etc.

The assessment of the apoptosis occurrence is another method of evaluating the quality of the embryo. The simplest method of the apoptosis assessment is morphological evaluation that allows to detect specific features of apoptosis at the level of nucleus morphology or degradation of DNA in the nucleoplasm (use of electron microscopy, fluorescent dyes, annexin-V, TUNEL assay).

Current methods

Among current methods of assessment there is **preimplantation genetic diagnosis**, which allows the detection of chromosomal abnormalities, defects of individual genes and sex determination in case of sex-related diseases (De Vos and Van Steirteghem, 2001).

Furthermore, this group includes **time-lapse technology** that allows monitoring of preimplantation embryo development from the zygote to the blastocyst without removal from the incubator (Payne et al., 1997).

The last group is represented by so-called **"Omics technologies"** that allow you to monitor the molecular composition of the embryo and interactions between the embryo and the surrounding culture medium. These interactions may represent (non-morphological) markers of embryo viability (Uyar and Seli, 2014). This group includes genomic, transcriptomic, proteomic and metabolomic analyses as well as analysis of small non-coding DNA.

It is very important to choose an appropriate method for evaluating the quality of embryos. When the selected embryo is to be used for the embryo transfer, it is necessary to use non-invasive methods of assessment. On the other hand, invasive methods provide more precise information on the viability of the embryo.

Evaluation of ejaculate quality

The ejaculate represents the secretion of male sexual organs, which is released during ejaculation reflex (Kováčik et al., 2009). It consists of the sperm fraction and the plasma fraction. The ejaculate assessment is especially important at the selection of males with good fertility and sperm quality for the production of sperm insemination doses (Dhurvey et al., 2012; Molinari et al., 2013).

Because fertilization is a complex process, it is not possible to predict the result of fertilization only by the application of single test to assess semen quality. Basic laboratory evaluation of the ejaculate includes macroscopic (volume, color, odor, consistency, miscellany, pH) and microscopic (total activity, progressive movement of sperm, agglutination, concentration, percentage of sperm morphological malformations, number and type of microbial germs) examination. In recent years, however, new much more precise methods of sperm analysis have come to the fore (De Pauw et al., 2003).

CASA assay

Computer-assisted sperm analysis (CASA) is a semi-automatic technology providing information on the sperm motility (Agarwal et al., 2008). This method evaluates the movement of individual sperm cells and captures digital images of their tracks. The resulting data are processed and subsequently used to observe kinetic properties of the sample (Fillers et al., 2008). The system is equipped with a phase-contrast microscope with a heating pad. The microscope is attached to the camera and the computer. Thea-Clément et al. (1996) reported the evaluation of the following motion parameters:

- % motile sperm = number of motile sperm/ total number x 100,
- VCL = curvilinear velocity ($\mu\text{m/s}$),
- VSL = straight-line velocity ($\mu\text{m/s}$),
- VAP = average path velocity ($\mu\text{m/s}$),
- LIN = linearity of motion (VSL/VCL),
- STR = straightness of motion (VSL/VAP),
- % progressively moving sperm = percentage of sperm with VAP > 40 $\mu\text{m/s}$ and straightness > 80 %,
- ALH = amplitude of lateral head displacement (μm),
- BCF = beat cross frequency (crossing/s).

The accuracy of this method is mainly affected by the concentration of spermatozoa, frame rate and sample preparation (Agarwal et al., 2008). Information obtained by CASA method is used to assess the impact of motility and motion characteristics of sperm cells on in vivo fertilization. However, this method is used for the evaluation of sperm samples only in a small extent, because the system is an intricate and needs the correct settings for each individual sample, in particular when the samples are derived from different species (Gillan et al., 2008).

Fluorescent techniques

Fluorescence is a member of the ubiquitous **luminescence** family of processes in which susceptible molecules emit light from electronically excited states created by either a physical (for example, absorption of light), mechanical (friction), or chemical mechanism. Generation of luminescence through excitation of a molecule by ultraviolet or visible light photons is a phenomenon termed **photoluminescence**, which is formally divided into two categories, **fluorescence** and **phosphorescence**, depending upon the electronic configuration of the excited state and the emission pathway.

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and subsequently to emit light of longer wavelength after a brief interval, termed the fluorescence lifetime. The process of **phosphorescence** occurs in a manner similar to fluorescence, but with a much longer excited state lifetime.

Fluorescent techniques are biophysical methodologies that exploit the phenomenon of fluorescence to examine and analyse protein–protein, protein–nucleic acid, ligand–receptor, and ligand–lipid interactions. These techniques are also useful in the study of protein conformation and orientation, as well as diffusion and binding constants.

Fluorescent techniques include, for example, flow cytometry, fluorescence microscopy, fluorescence-activated cell separation, time-resolved fluorescence, fluorescence polarization, fluorescence resonance energy transfer, multiphoton microscopy or fluorescence correlation spectroscopy. A basis for all fluorescent methods lies in the ability of marker molecules to emit radiation of electrons in the excited state, which are formed by absorption of radiation. The molecules can

absorb radiation of different wavelengths depending on the configuration of electrons, then pass into the excited state, and after a brief transition to the singlet state they are returned back to the baseline condition. A later transition, appearing after a short interval, is called a lifetime of fluorescence. It is linked to emission of radiation of a longer wavelength. The difference between the energy of emission and energy of excitation is called Stokes shift, which represents a unique capability of all fluorochrome molecules (Ellers and Johnson, 2010).

To observe the presence of biomolecules in cells and in other systems of biological origin the fluorescent agents are used. When choosing a fluorescent probe to assess the characteristics of sperm it is important to consider many factors, including, for example photostability, biological functions, emission and excitation wavelength of fluorescence, fluorescence lifetime, size and quantum yield (Petrunina and Harrison, 2013).

Fluorescent microscopy

Fluorescence microscopy and its improved version - confocal microscopy are the methods often used to assess sperm features, such as integrity of the acrosome, membrane integrity, calcium uptake and interaction with the female reproductive tract, and ova or protein phosphorylation (Rath et al., 2006). The advantage of fluorescence microscopy is an ability to identify topographical localization of the fluorescent signal in an individual cell using system of filters and mirror in the fluorescent microscope. The level of fluorescence can be quantified at the cellular level. Assuming that the noise of the detector and the optical background is low enough, it is also possible to detect emissions from the individual molecule of a fluorochrome (Petrunina and Harrison, 2013).

Flow cytometry

This is a process at which fluorescently-labeled cells at high speed pass separately through a flow membrane, where they are illuminated by lasers, resulting in light diffusion and fluorescence excitation of markers, which are located on certain parts of the sperm. The

markers are recorded by a photodetector and sent to a computer program, which processes an information in the units of fluorescence intensity, which are usually displayed in form of dispersed areas or a histogram (Martinez-Pastor et al., 2010). During the analysis of sperm by flow cytometry various problems occur, in particular presence of foreign particles in the sample. There are the immature forms of spermatogenic cells, blood cells, tissues, bacteria and various impurities occurred following semen thawing. Such foreign particles can distort the results on a scatterplot during data analysis (Dhurvey et al., 2012).

Using flow cytometry it is possible to examine thousands of sperm in seconds. The disadvantage of this method is a high cost at the wider application of this method in the laboratory. The method also applies fluorescent probes used for sperm staining and helps to increase the objectivity of the analysis of a large number of sperm. Flow cytometry evaluates several characteristics of sperm, and is able to provide a specific description of the population of sperm (Dhurvey et al., 2012).

5. Sample preparation and evaluation by electron microscope

Electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing to see much smaller objects in finer detail. Optical microscopes can magnify between 40 to 2000 times but the electron microscope can resolve features that are more than 1 million times smaller.

Today there are two major types of electron microscopes used in clinical and biomedical research settings: the transmission electron microscope (TEM) and the scanning electron microscope (SEM):

- TEM magnifies 50 to ~1 million times,
- SEM magnifies 5 to ~ 500,000 times.

As compared with the processing of the samples for evaluation under light microscopy, the sample preparation for electron microscopy evaluation differs in the processes by which the sample is running, and also in the way of its placement. For each type of microscopy a sample preparation procedure is different. The electron microscope contains a vacuum, which is unfavorable for biological preparations. The water located in the sample, at a high vacuum in the microscope, can evaporate rapidly and lead to a collapse in the sample structure and to the sample drying. If we want to observe 'biological samples in their native state, we have to prepare them before putting into the microscope.

Therefore, biological sample can be prepared either by removing or by freezing water. The oldest method is a conventional sample preparation using chemical fixation, sample dehydration at room temperature and embedding into a chosen resin. In the 1970s,

Tokuyasu introduced an alternative approach to conventional sample preparation dedicated for immunocytochemistry. This alternative method to chemical fixation is a cryofixation via vitrification process.

Technique of sample preparation for electron microscopy assay (chemical fixation)

Preparation and processing of samples for transmission electron microscopy evaluation is a complex process consisting of a number of sequential steps. Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The choice of proper technique depends on the type of specimen.

Biological material (oocytes, embryos, sperm and stem cells) for transmission electron microscopy evaluation is prepared according to the following protocol:

- 1) Fixation in the Karnowski´ fixative (2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 mol l⁻¹ cacodylic buffer, pH 7.1 to 7.3) at 4 °C for 1 hour, and washing in a cacodylic buffer.
- 2) Post-fixation in 1% solution of osmium tetroxide in cacodylic buffer for 1 hour.
- 3) Embedding into the blocks of 4% agar.
- 4) Dehydration of the samples by incubation in a sequential set of increasing concentrations of acetone (30, 50, 70, 90% and absolute acetone).
- 5) The oversaturation of the dehydrated samples and embedding into a Durcupan ACM embedding resin (Fluka, Buchs, Switzerland) in a silicone mold.
- 6) The hardening for 48 hours at 60 °C.
- 7) Cutting of semi-thin sections (500 nm) and their staining with a Toluidine blue.
- 8) Cutting of ultra-thin serial sections (70 nm) by using the Ultracut UC 6 (Leica) ultramicrotome and their application to a nickel mesh of 150 grade.
- 9) the contrasting of the sections with 10% solution of uranyl acetate in absolute methanol for 30 min, and in lead citrate for 3 min according to Reynolds (1963).

- 10) Evaluation of sections under a transmission electron microscope JEM 100 CX II, (Jeol, Japan) at an accelerating voltage of 80 kV, and preparation of electronograms.
- 11) Evaluation of the ultrastructure of cell organelles (nucleus, nucleoli, mitochondria, endoplasmic reticulum, Golgi apparatus, lipids, vacuoles, inclusive and apoptotic bodies) from the electronograms (Dragin et al., 2006).

Ultrastructure of cell organelles

The term “ultrastructure” means detailed structure of biological samples such as cells (Figs. 4.1 and 4.2), tissues and whole organs, which are observed under an electron microscope. The quality of the biological material is characterized by the ultrastructure of individual cell organelles.

Fat (lipid) droplets – belong to the inclusion bodies of the cell and occur in the form of droplets of different sizes. The fat reduces osmium oxide, which is used for fixing at the electron microscopy, on metallic osmium, and that is why the fat droplets appear under the electron microscope as black circular objects. The synthesis and accumulation of lipids takes place in the cytoplasm of cells but not in the organelles, and therefore these cell inclusions do not have membranes on their surface (Belak, 1990). Lipid droplet size varies from 0.1 to 1.6 microns, and during the period of embryonic development their number increases (Belak, 1990).

Rough endoplasmic reticulum (RER) – is a system of cisterns formed by membranes, on which ribosomes and polyribosomes are bound. Under the electron microscope RER has a grainy appearance. Individual cisterns of the reticulum communicate with each other (Belak, 1990). In this type of reticulum, the most important is an enlargement of the space between the individual membranes. This expansion causes the formation of vacuoles or vesicles. Too much dilatation of reticulum tubules leads to cell death. This pathological deviation is associated with the disconnection of the ribosome from the reticulum membrane and is caused by the accumulation of fluid in the vacuoles (Massányi et al., 2004). Granular membranes (porous cisterns) arise in the reticulum as pathological reduplicates of the nu-

clear membrane and imitate the pores of a karyolemma. This negative phenomenon is observed in the embryonic cells and tumor cells (Bózner, 1992).

Golgi apparatus – a system formed by smooth membranes, which together create a certain number of cisterns, vesicles and vacuoles. In terms of functions, its convex side with their vesicles confirms close functional association with the rough endoplasmic reticulum. The concave side of the Golgi apparatus is characterized by vacuoles containing secretory products. The main function of the Golgi apparatus is both regulated and unregulated secretion. The increase in their activity starts with the hyperplasia, while the decrease starts with the atrophy (Bózner, 1992). The pathological mechanism depends on the presence of ATP and intracellular calcium (Massányi et al., 2004).

Mitochondria – semi-autonomous, ubiquitous components of cells, which are essential for metabolic and energy processes in the cell (Bózner, 1992). Their appearance is similar to the shape of fibers or beads, and their wall is formed by two membranes, spaced about 8 nm between them (Belak et al., 1990). The morphological signs of functional disorders of mitochondria include pathological localization, destruction, changes in the shape of cristas and expansion of their interior space. The lack of oxygen is manifested by hydration and swelling of mitochondria, but these changes at the first phases are even reversible to some extent. The volume of mitochondria may be increased due to the osmotic disorders, which destroy the mitochondrial matrix. Hypo-osmotic conditions lead to swelling of mitochondria, which in this state decrease their enzymatic activity (especially intracellular respiration). On the contrary, enlargement of mitochondria is parallel mainly with a reduced activity of phosphorylation mechanisms and with the disconnection of oxidative phosphorylation. Enlargement of mitochondria can be classified as compensation due to the chronic shortage of ATP. The physiological life of mitochondria is within the range of 10-12 days, but their lifetime is strongly influenced by pathological conditions (Bózner, 1992).

Nuclear membrane – is formed by two membranes (inner and outer), which are distanced from each other about 40-70 nm. The space between the membranes is called the perinuclear space and this can communicate with endoplasmic reticulum. The two-layer envelope is interrupted in some places of the karyolemma generat-

ing the nuclear pores, which contain granulosa material defined as a diaphragm. Due to the increased transport and the exchange of substances between the nucleus and cytoplasm, the nuclear surface increases (hypertrophy and cell growth). In the case of pathological conditions, there is a change in the size and number of nuclear pores, what may lead to the destruction of the karyolemma, broader junctions between the cytoplasm and karyoplasm, or their intermixing, especially in the cell death process (Bóznér, 1992).

Vacuoles – are organelles that occur mostly in plant cells, but they are also present in animal cells in a system similar to plant vacuoles. They play an important role in the osmotic processes of cells (Bóznér, 1992).

Microvilli – are thin, about 1-2 μm in length (Belak et al., 1990), 0.06 μm thick (Bóznér, 1992), finger-like projections of the cytoplasm, whose surface is surrounded by a cytoplasm membrane. The internal content of microvilli is formed of a homogenous and slightly dark cytoplasm with microfilaments, which are grouped into bundles. Such bundles extend along the microvilli and intervene into the cytoplasm of cells (Belak et al., 1990).

Cytoplasm membrane – covers the surface of cells, protects cells against environmental changes and provides communication between the cytoplasm and the outside (Massányi et al., 2004), and its thickness is about 7 nm (Belak et al., 1990). Special structural components of the cell membrane induce morphological changes, changes in permeability and function of the permeation and creation of new antigenic properties (Bóznér, 1992).

The **dense bodies** (lysosomes) – are specialized organelles of a cell, bounded by a membrane and formed in the Golgi apparatus. Dense bodies have a uniform electron-dense appearance and size of 0.03 - 1 μm . In these organelles an intracellular digestion and renewal of cell components takes place (Massányi et al., 2004).

Cellular debris – are located in the perivitelline space of the embryo as a product of cellular metabolism. The presence of a large number of extruded cells or their parts in the perivitelline space is considered as a sign of poor embryo quality. When fragmentation occurs extensively, it can have a disastrous effect on the embryo developmental potency (Popelková et al., 2005).

Flocculent (flake-like) vesicles – are cell formations bounded by biological membrane, and have a lipid-protein structure in the cytoplasm of cells.

Contact of two cells by the type of the “**gap junction**” – is the easiest way to connect two adjacent cells (Massányi et al., 2004). Cytoplasmic membranes of adjacent cells are not connected to each other, but create an open channel (Pivko, 1995). This type of connection is formed in the way that the space between the membranes of two cells (with a thickness of about 12-20 nm) is filled with mukopolysaccharides, which act as biological glue between them. In this connection, the presence of calcium and vitamin C is very important (Massányi et al., 2004). This type is specific for the connection of individual cells of embryoblast between each other or for the co-juncton of trophoblast and embryoblast cells. The channels of a one gap junction allow direct penetration of low molecular weight substances from cell to cell, and thus bypass the intracellular space (Pivko, 1995).

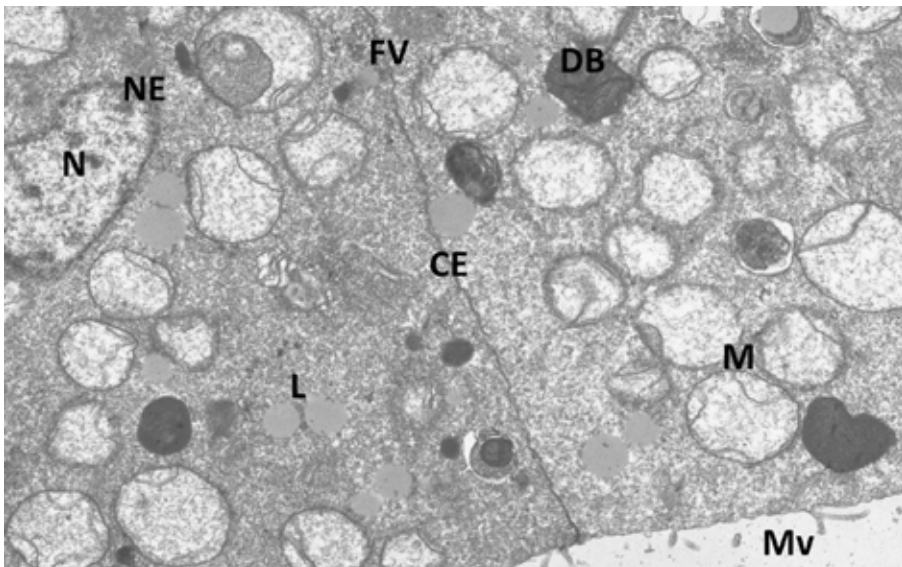


Figure 4.1 Micrograph of an ultrathin section through the rabbit embryo with organelles that showed pathological signs. Mv - microvilli, CE - cytoplasmic membrane M - mitochondria, NE - nuclear membrane, N - nucleus, FV - flocculent vesicles, L - fat droplets, DB - dense bodies.

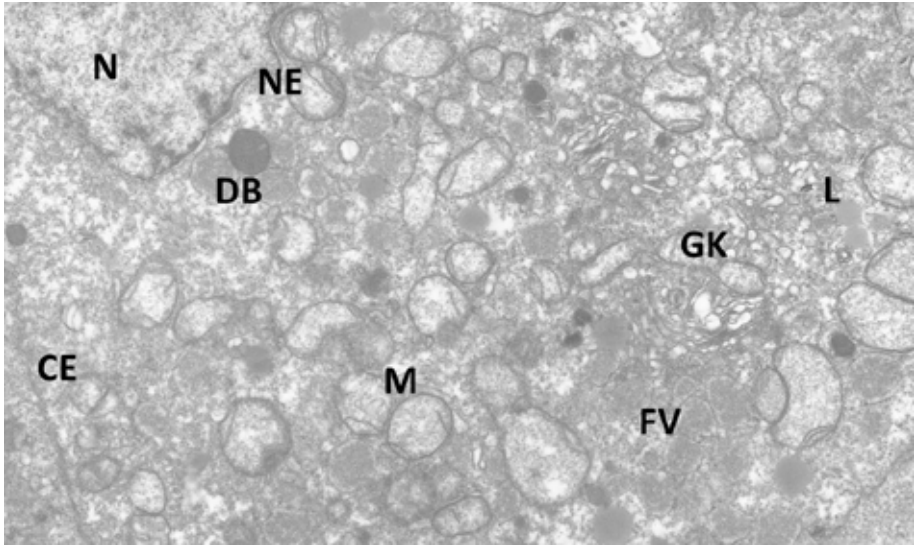


Figure 4.2 Micrograph of an ultrathin section of the rabbit embryo with normal organelles. M - mitochondria, CE - cytoplasmic membrane, NE - nuclear membrane, N - nucleus, FV - flocculent vesicles, L - fat droplets, DB - dense bodies, GK - Golgi complex.

6. Ultrastructure of rabbit oocyte

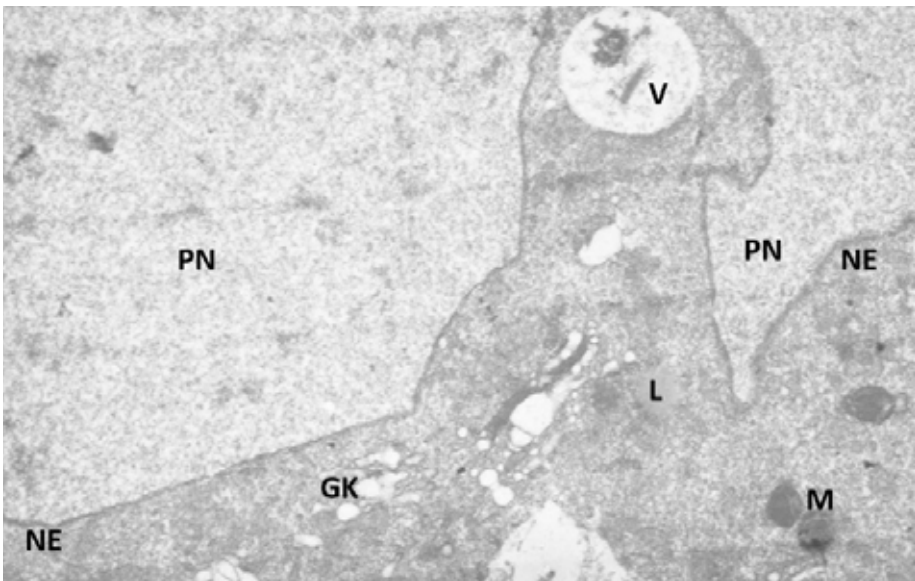


Figure 6.1 A fertilized oocyte-zygote of rabbit at 16 hours after mating, at stage of two pronuclei, paternal and maternal (pronucleus - PN), with a corrugated surface and undulating pronuclear membranes (NE), characteristic of the beginning of the syngamy process. In close proximity to the Golgi complex (GK), vacuole (V), mitochondria (M) and lipid droplets (L) are visible. TEM, x10 000.

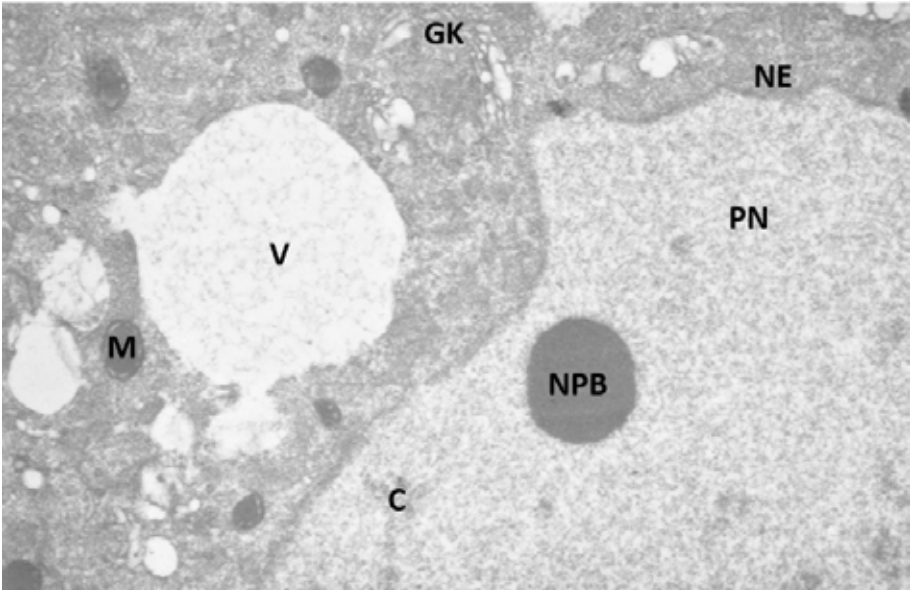


Figure 6.2 Rabbit zygote at stage of the syngamy of two pronuclei. A part of one pronucleus (PN) with the nucleolus precursor body (NPB), and condensed chromatin (C). Undulating pronuclear membrane (NO), the Golgi complex (GK), mitochondria (M), vacuole (V). TEM, x10 000.

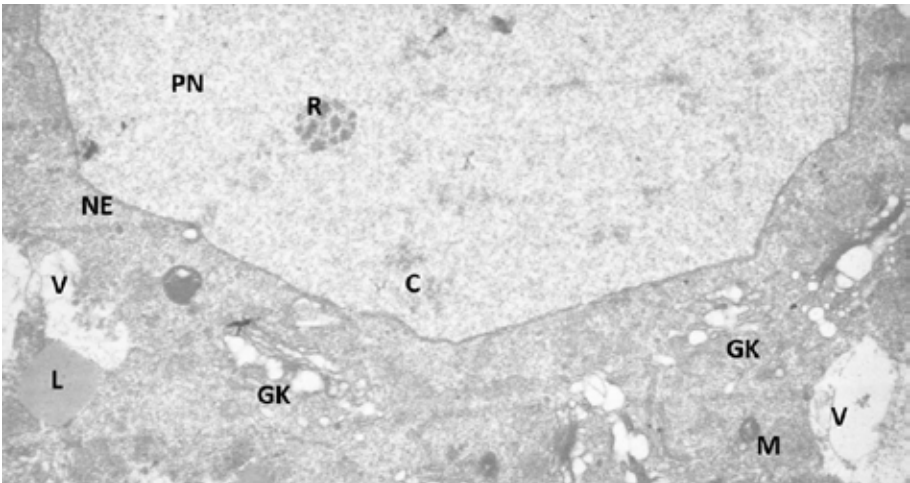


Figure 6.3 Rabbit zygote at stage of the syngamy of two pronuclei. A part of the second pronucleus (PN) with the nuclear precursor body, which is transformed into an electron-dense reticulum (R) and condensed chromatin (C). In the vicinity of the pronucleus there are Golgi complex (GK), mitochondria (M), the fat droplets (L) and vacuoles (V). Undulating membrane of the pronucleus (NE). TEM, x10 000.

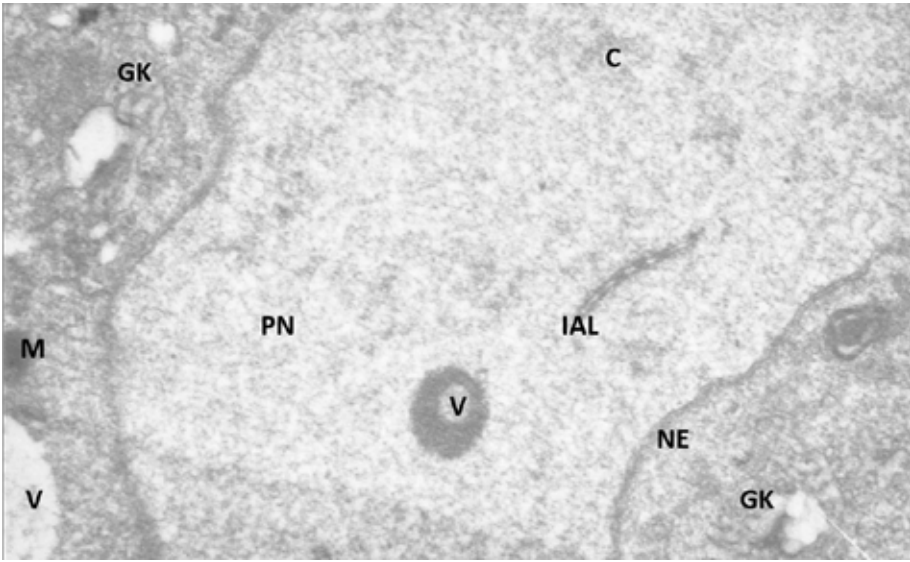


Figure 6.4 Pronucleus (PN) with the nuclear precursor body with a central vacuole (V) and intrapronuclear annulate lamellae (IAL) and condensed chromatin (C). In the vicinity of the pronucleus there are Golgi complex (GK), mitochondria (M) and vacuole (V). Undulating membrane of the pronucleus (NE). TEM, x10 000.

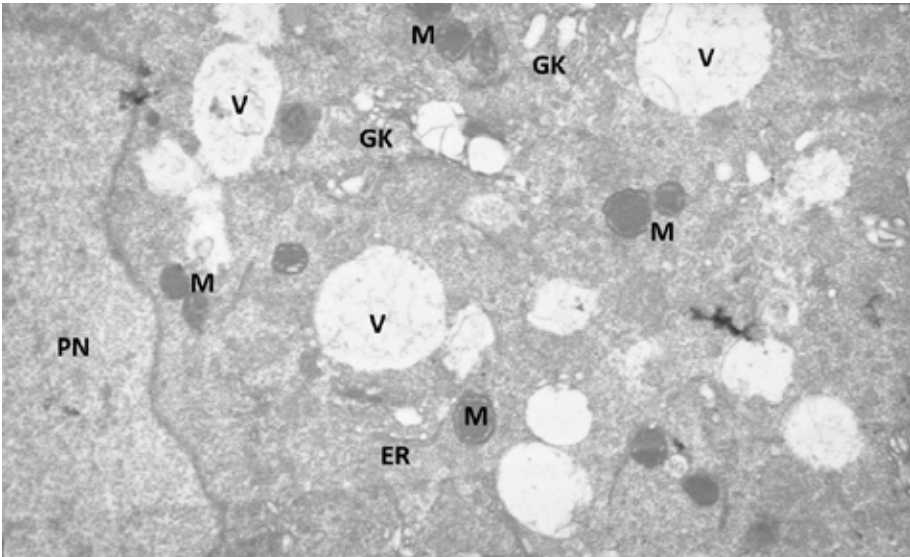


Figure 6.5 The occurrence of numerous mitochondria (M) and vacuoles (V) close to the pronucleus (PN). They are also clusters of the Golgi apparatus (GK) and small vesicles of the endoplasmic reticulum (ER). TEM, x10 000.

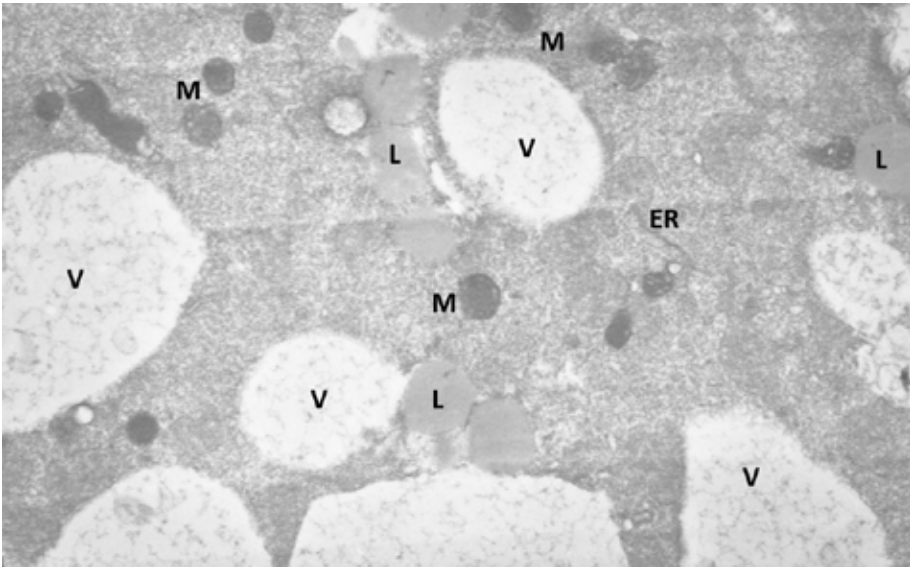


Figure 6.6 Rabbit zygote at stage of the syngamy of two pronuclei. Numerous large vacuoles (V), fat droplets (L), electron-dense mitochondria (M) and the endoplasmic reticulum (ER).
TEM, x10000.

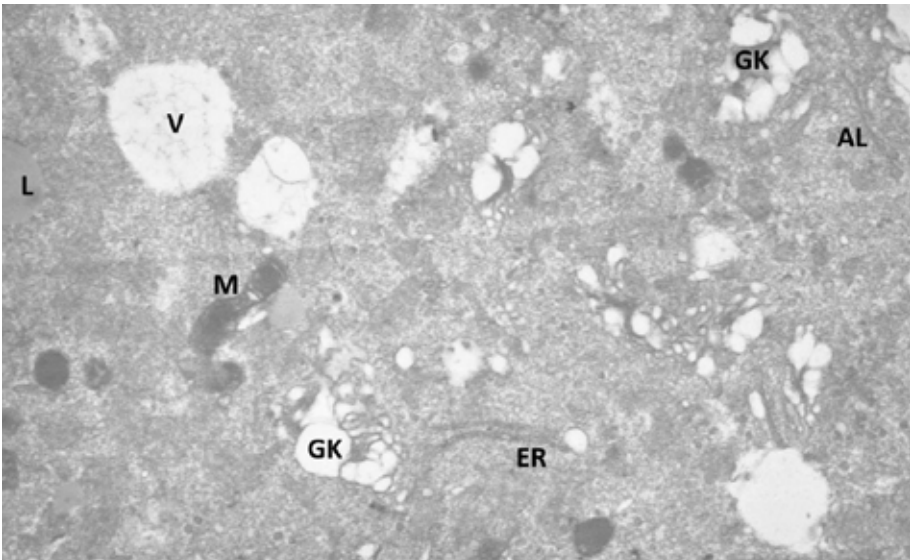


Figure 6.7 Vesicles of Golgi complexes (GK), the vacuole (V), mitochondria (M), the fat droplets (L), the cytoplasmic annulate lamellae (AL), and small vesicles of endoplasmic reticulum (ER) in the ooplasm of rabbit zygote.
TEM, x10000.

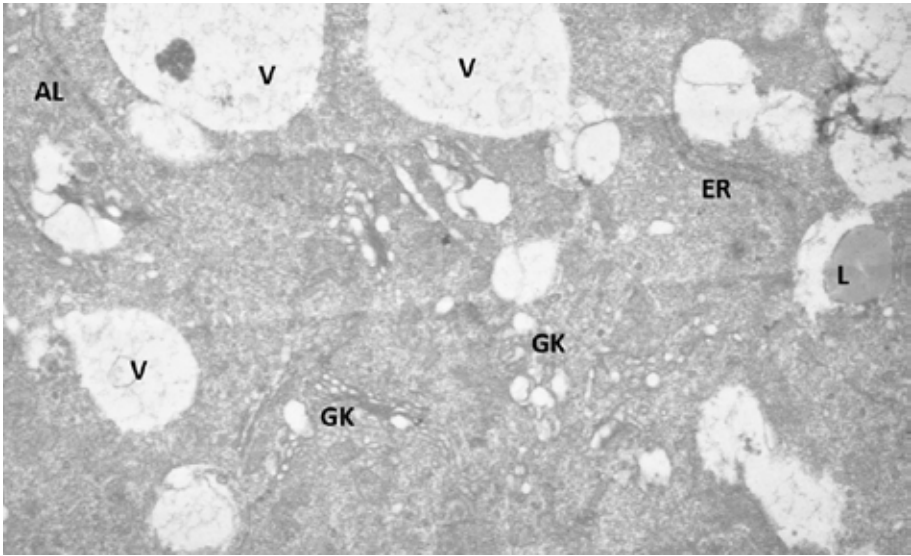


Figure 6.8 Occurrence of clusters of annulate lamellae (AL) in the ooplasm around a one of the pronuclei of rabbit zygote. Numerous Golgi complexes (GK), endoplasmic reticulum (ER), vacuoles (V) and the lipid droplet (L). TEM, x10 000.

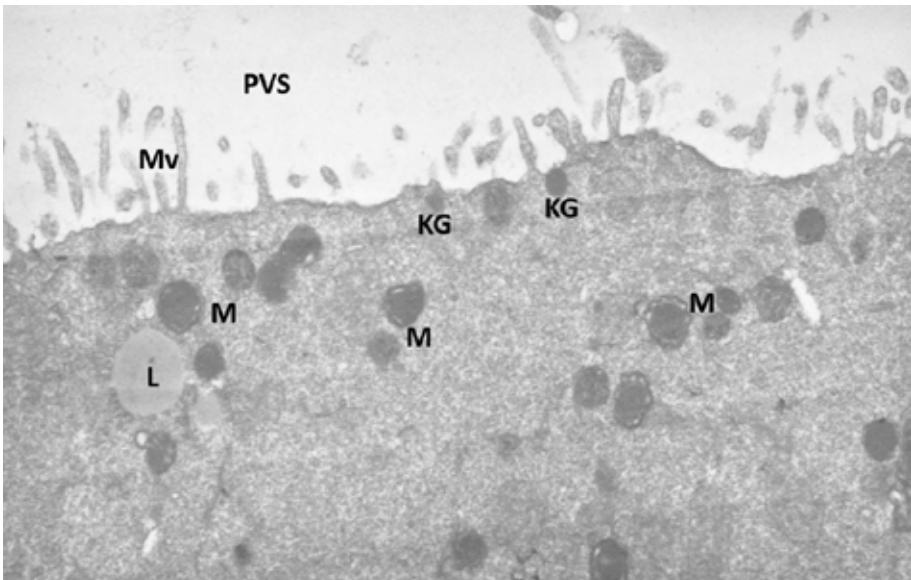


Figure 6.9 Numerous clusters of dense oval mitochondria (M) in the cortical region of a rabbit zygote. Rare occurrence of cortical granules (KG) and lipid droplets (L). Microvilli (Mv) of the plasma membrane are oriented toward the perivitelline space (PVS). TEM, x10000.

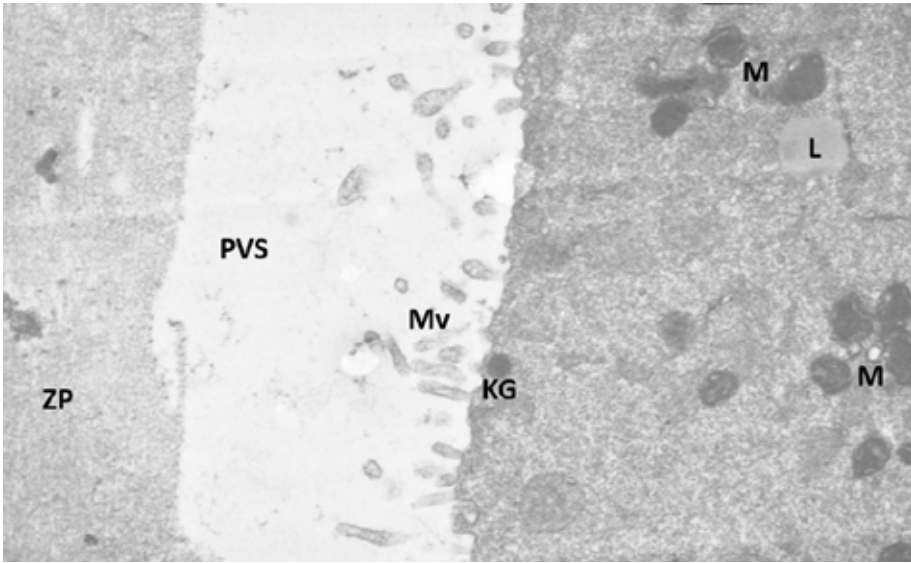


Figure 6.10 Microvilli (MV) are oriented toward the perivitelline space (PVS) under the zona pellucida (ZP). The dense oval mitochondria (M) in the cortical region of a rabbit zygote. Rare occurrence of cortical granules (KG) and lipid droplets (L). TEM, x10000.

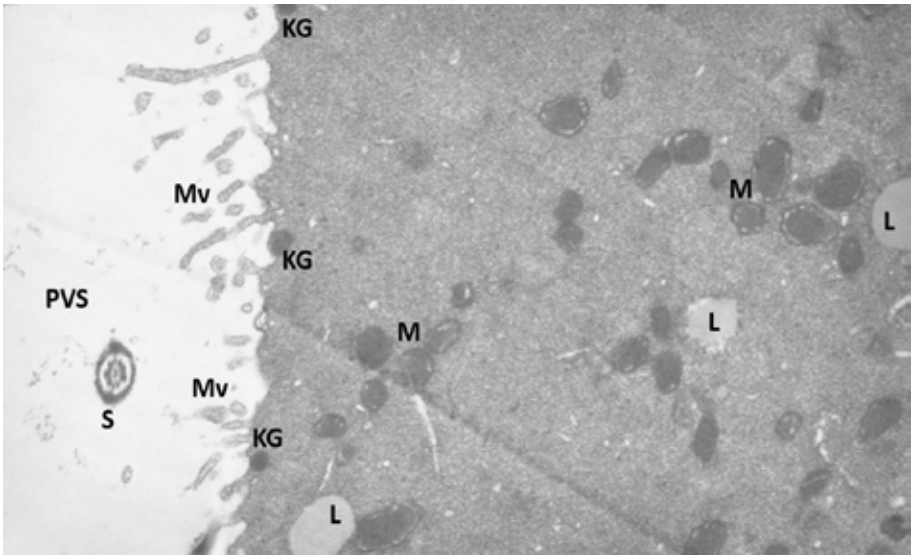


Figure 6.11 Cross-section along the main region of the rabbit sperm tail (S) in the perivitelline space (PVS) over the microvilli (Mv). Solitary formation of cortical granules (KG) in contact with the membrane of the zygote, the lipid droplets (L), dense oval mitochondria (M) in the cortical region. TEM, x10000.

7. Ultrastructure of rabbit sperm

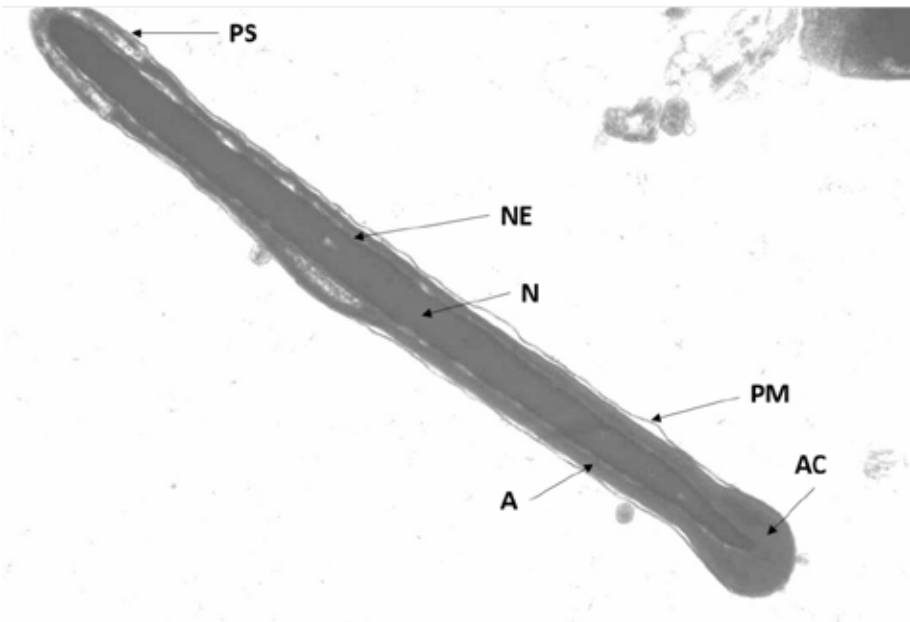


Figure 7.1 Diagonal section in the longitudinal axis of the rabbit sperm head. The nucleus (N, nucleoplasm) markedly narrowed toward the apical portion, the nuclear envelope (NE), acrosomal matter (A), apical ridge (AC), plasma membrane (PM), post-acrosomal sheath (PS). TEM, x14000.

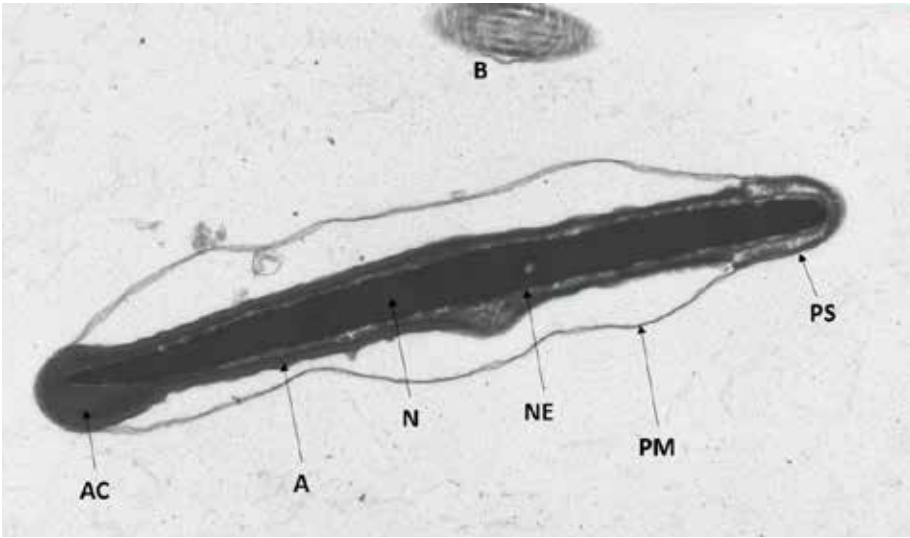


Figure 7.2 Diagonal section of rabbit sperm head. The nucleus (N, nucleoplasm) markedly narrowed toward the apical part, nuclear envelope (NE), acrosomal matter (A), part of the apical ridge (AC), wavy plasma membrane (PM), post-acrosomal sheath (PS), diagonal section along the main region of the sperm tail (B). TEM, x14000.

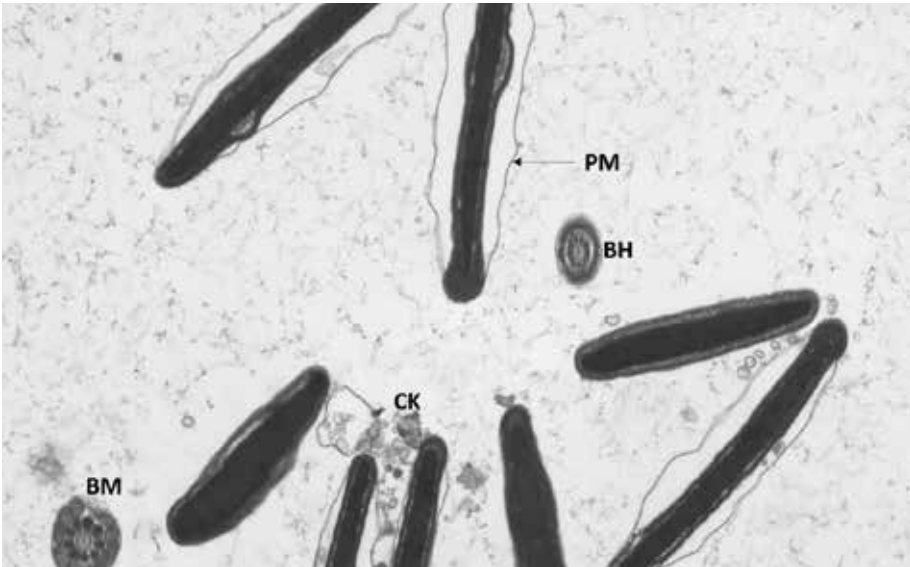


Figure 7.3 Diagonal section of the rabbit sperm head. The waved plasma membrane (PM), remnants of the cytoplasmic droplet (CK), a cross-section of the mitochondrial segment of the flagella (BM), a cross-section of the main section of the flagella (BH). TEM, x7200.

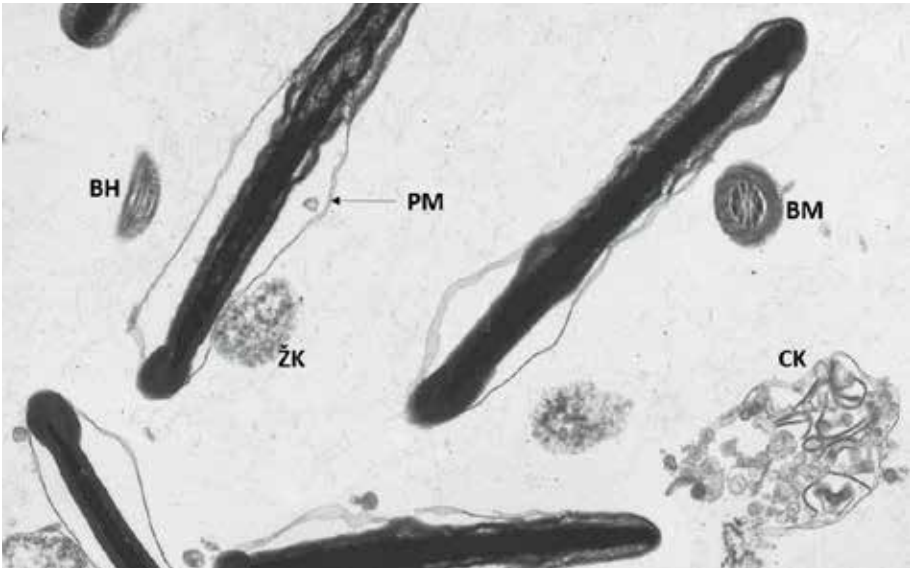


Figure 7.4 Diagonal section of rabbit sperm heads. The undulating plasma membrane (PM), cytoplasmic drop (CK), a cross-section of the mitochondrial segment of flagella (BM), a cross section of the main segment of flagella (BH), the yolk droplets (ŽK). TEM, x7 200.

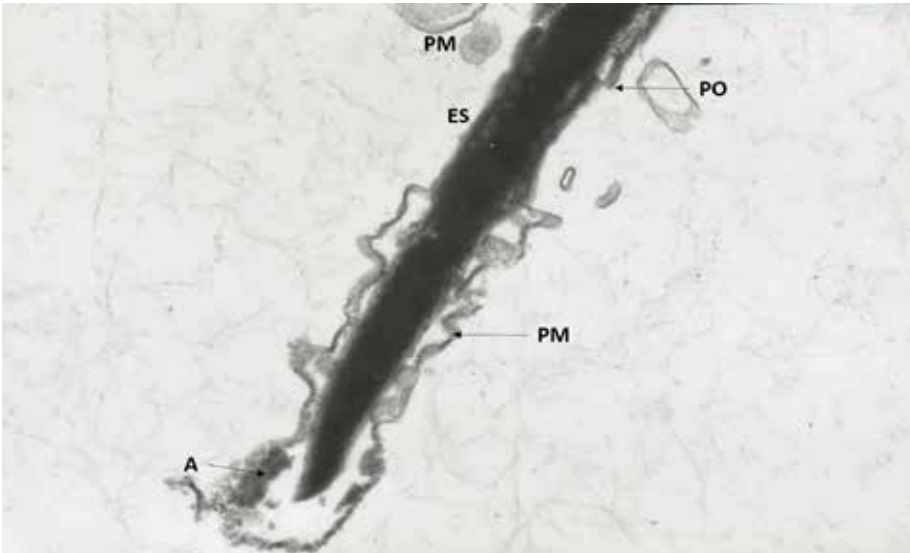


Figure 7.5 Apical portion of the rabbit sperm head with swollen acrosome. Remnants of disintegrated acrosomal matter (A) and decrease in its density, waved plasma membrane (PM), the area of the equatorial segment (ES), post-acrosomal edge (PO), residues of the plasma membrane (PM). TEM, x14 000.

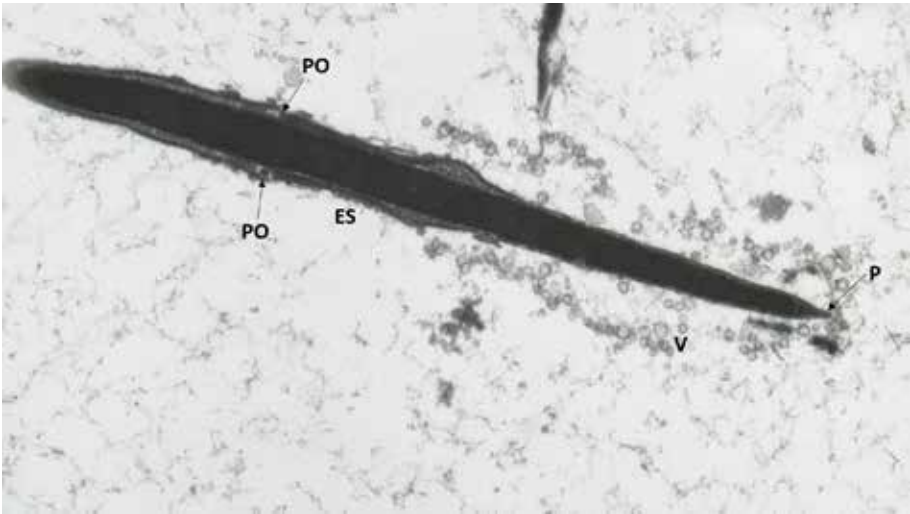


Figure 7.6 Apical portion of the rabbit sperm head with the acrosomal reaction. Vesicles (V) of a relative stable size and the loss the acrosomal matter, clearly visible perforatorium (P) on the apical part of the nucleoplasm, the area of the equatorial segment (ES), post-acrosomal edge (PO). TEM x14 000.

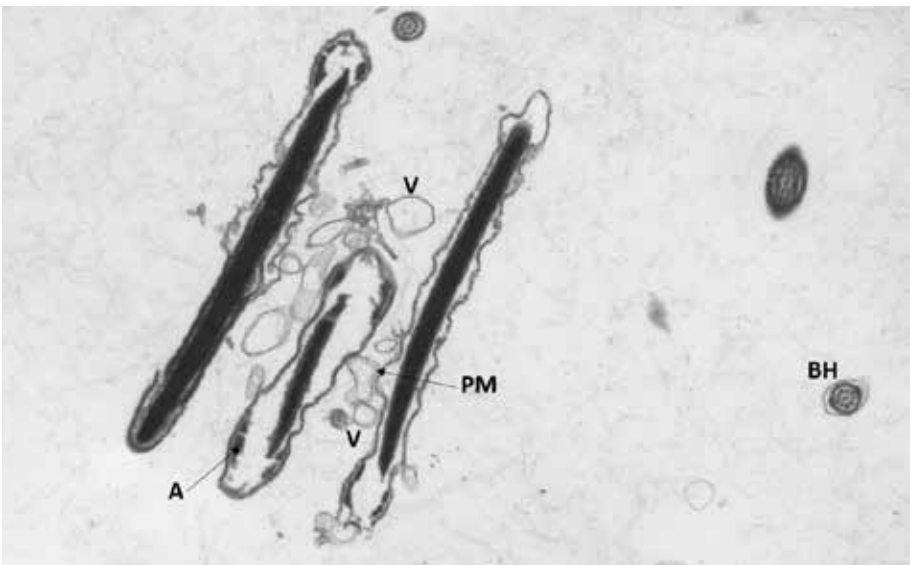


Figure 7.7 A group of three sperm heads connected by slips of plasma membranes. The vesicles of the plasma membrane (V) of different size, specific for the acrosomal reaction, coupling of the plasma membrane (PM) and the outer acrosomal membrane, residues of the acrosomal matter (A), a cross-section of the main section of flagellum (BH). TEM, x7 200.

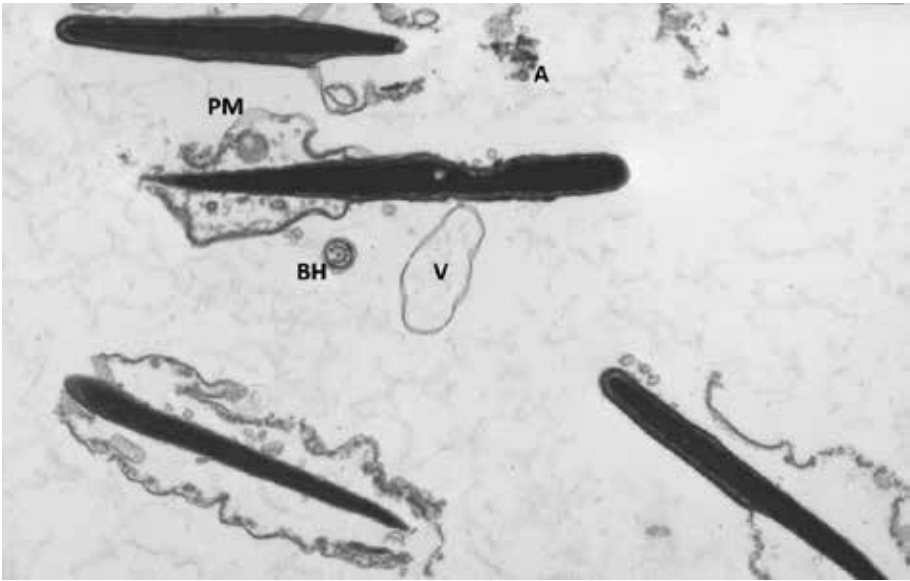


Figure 7.8 A group of four rabbit sperm heads. The vesicles (V) of the plasma membrane at the pseudoacrosomal reaction, coupling of the plasma membrane (PM) and the outer acrosomal membrane, residues of the acrosomal matter (A), in the center there is a cross-section of the main section of flagellum (BH) without microtubules, disorder known as Dag defect. TEM, x7 200.

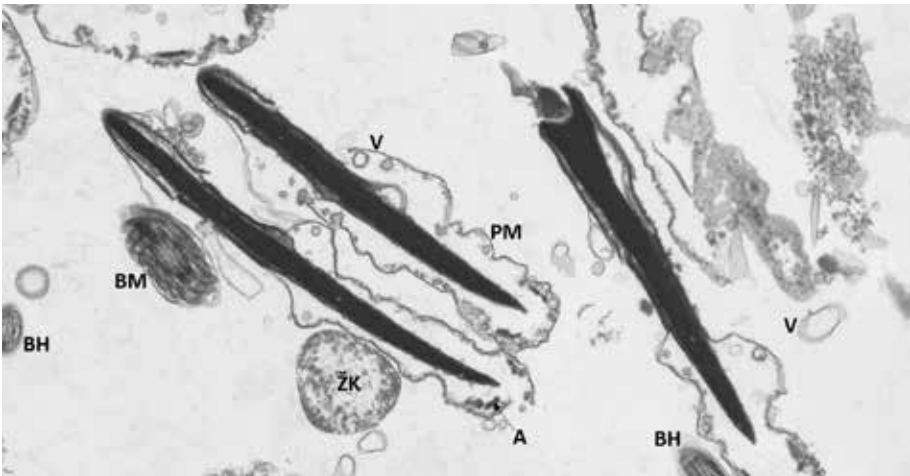


Figure 7.9 In the middle there is a group of two merging heads of rabbit sperm. Vesicles (V) of the plasma membrane at the pseudoacrosomal reaction, scraps of the connection between the plasma membrane (PM) and outer acrosomal membrane, remnants of the acrosomal matter (A), diagonal section of the mitochondrial compartment of flagellum (BM), main segment of flagella (BH), the yolk droplets (ŽK). TEM, x7 200.

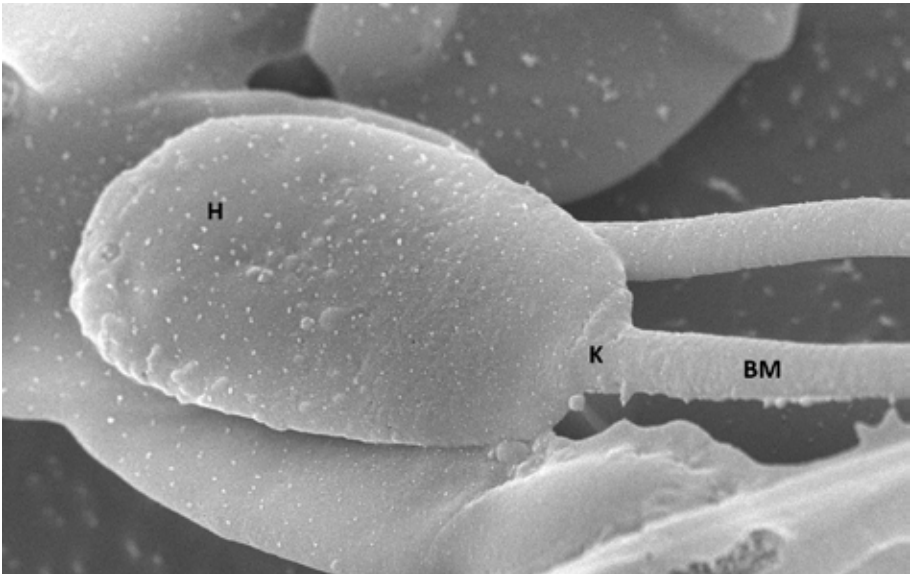


Figure 7.10 Rabbit sperm from fresh semen ejaculate. The sperm head (H) with the neck (K) and a portion of mitochondrial section of the flagella (BM). Cryo-SEM, x10 000.

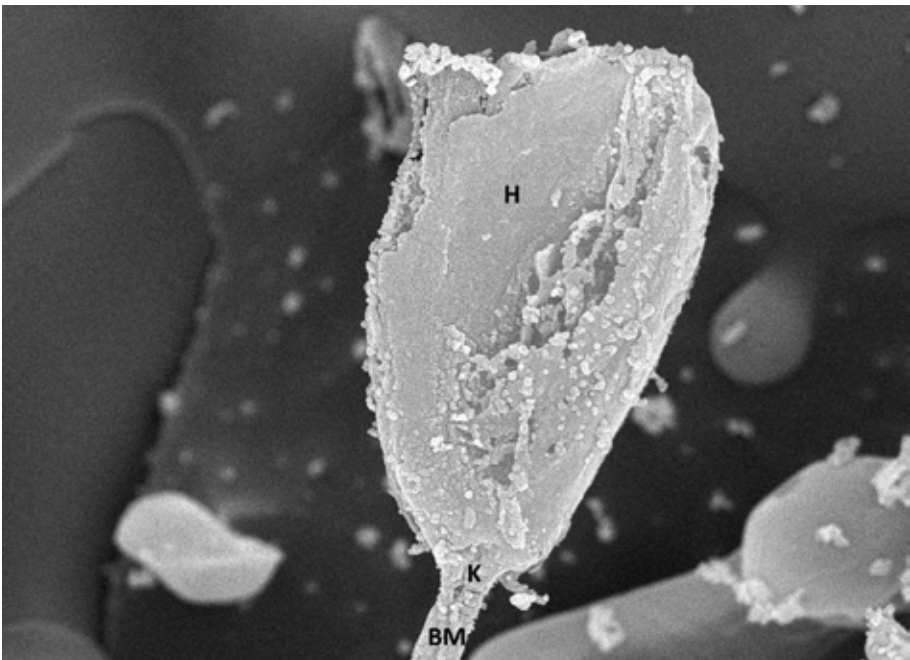


Figure 7.11 Sperm cell from cryopreserved rabbit ejaculate. The sperm head (H) with the neck (C) and a portion of the mitochondrial section of flagella (BM) damaged by cryopreservation. Cryo-SEM, x10 000.

8. Ultrastructure of rabbit embryos

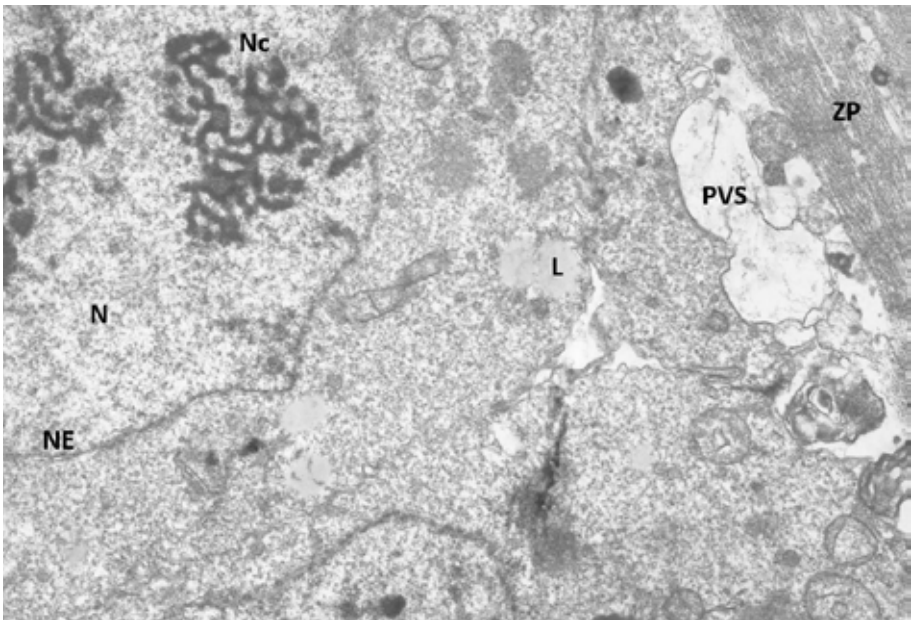


Figure 8.1 Rabbit embryo at the stage of compact morula. Nucleus (N), large nucleolus with nucleolonemes (Nc), nuclear envelope (NE), lipid droplets (L) perivitelline space (PVS), zona pellucida (ZP). TEM, x7 200.

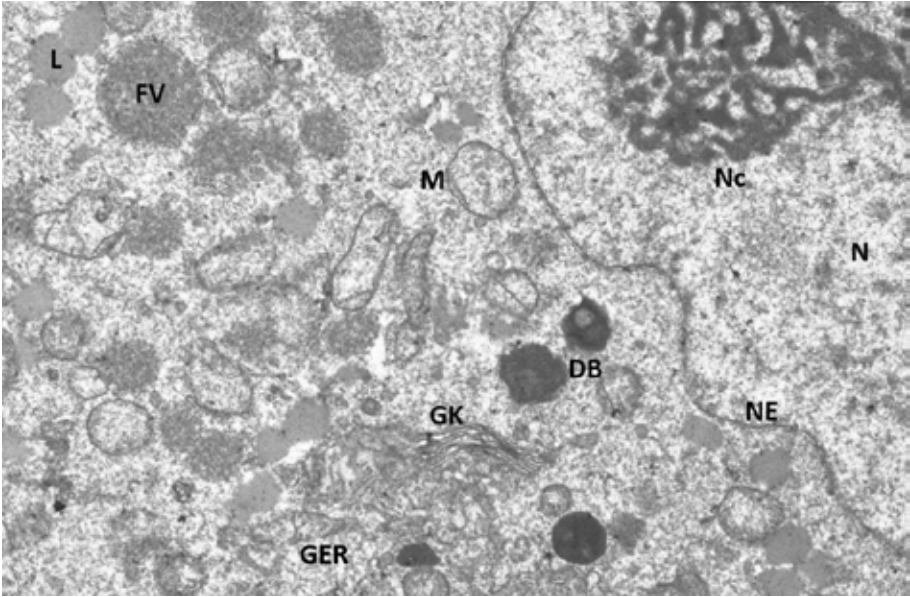


Figure 8.2 Rabbit embryo at the compact morula stage. The nucleus (N), nucleolus with nucleolonemes (Nc), the nuclear envelope (NE), the Golgi complex (GK), short sections of granulated endoplasmic reticulum (GER), mitochondria (M), lipid droplets (L), vesicles with a flocculent material (FV), dense bodies (DB). TEM, x7 200.

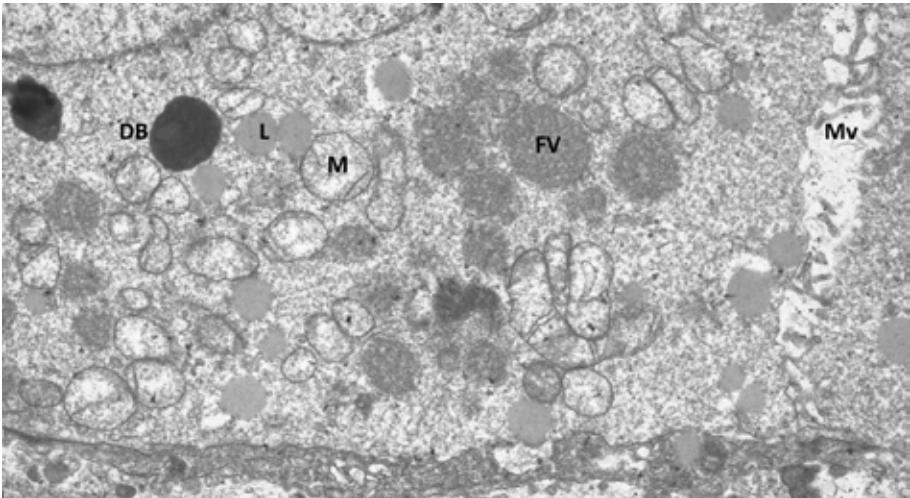


Figure 8.3 Rabbit embryo at the morula stage. Microvilli among the blastomeres in the perivitelline space (Mv), mitochondria (M), lipid droplets (L), vesicles with a flocculent material (CM), the dense bodies (DB). TEM, x7 200.

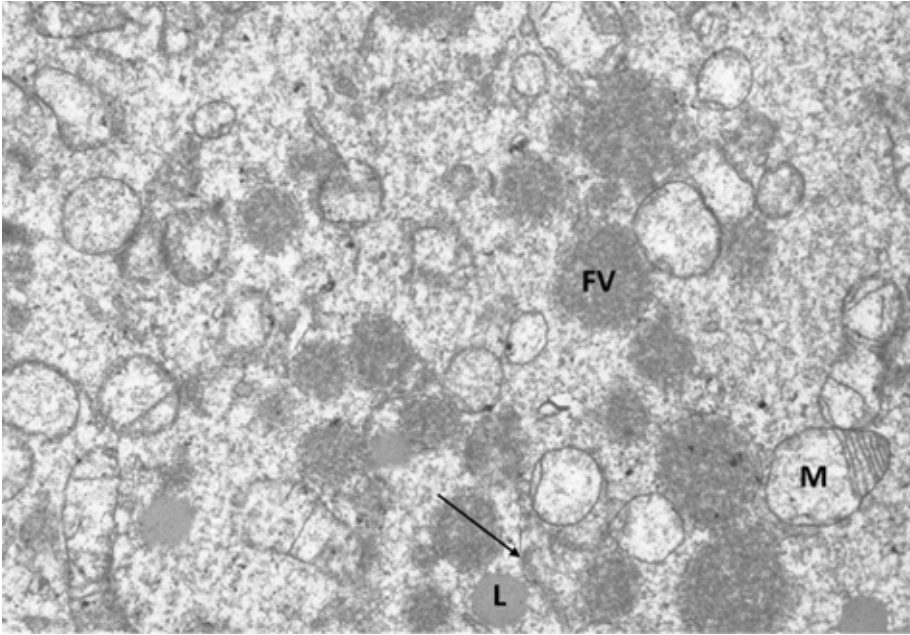


Figure 8.4 Rabbit embryo at the morula stage. A group of different sized vesicles with a flocculent material (FV) and in close proximity to the mitochondria (M) and membranes of the granulated endoplasmic reticulum (arrow), lipid droplets (L). TEM, x7 200.

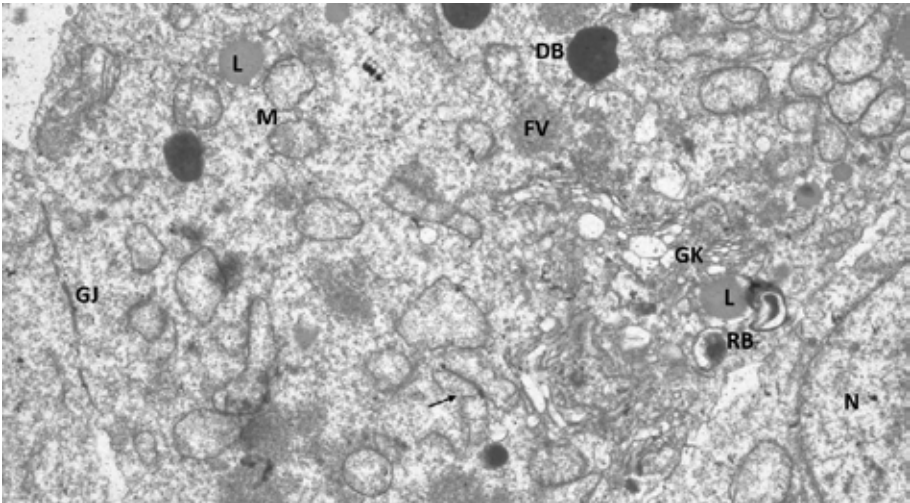


Figure 8.5 Rabbit embryo at the compact morula stage. The nucleus (N), the Golgi complex (GK), a group of short cisterns of granulated endoplasmic reticulum (arrow), mitochondria (M), lipid droplets (L), vesicles with a flocculent material (FV), the dense bodies (DB), residual bodies (RB), intercellular communications "gap junction" (GJ). TEM, x7 200.

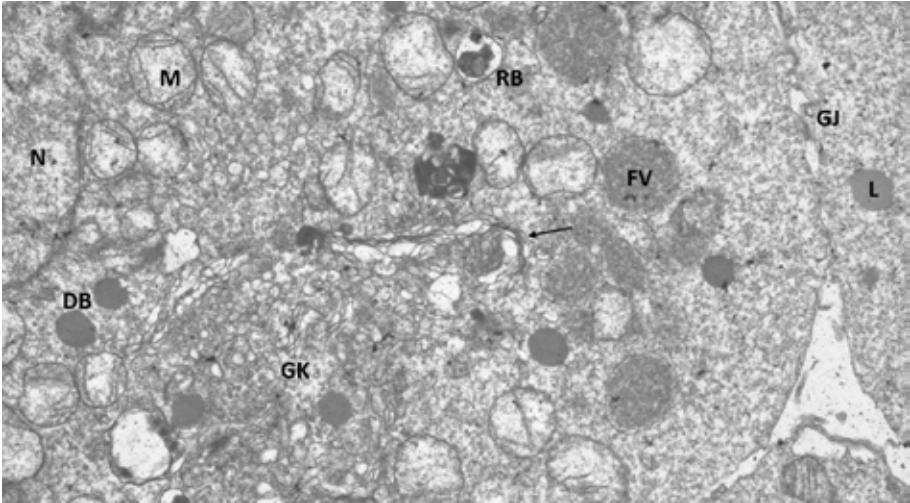


Figure 8.6 Rabbit embryo at the compact morula stage. The nucleus (N), the Golgi complex (GK), granulated endoplasmic reticulum (arrow), mitochondria (M), lipid droplets (L), vesicles with a flocculent material (FV), the dense bodies (DB), residual bodies (RB), intercellular communications “gap junction” between neighboring blastomeres (GJ). TEM, x7 200.

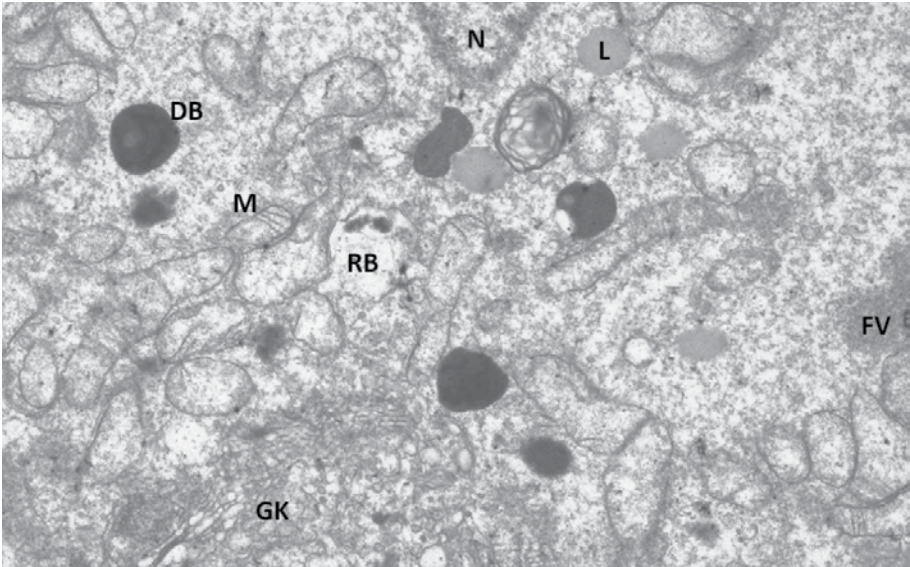


Figure 8.7 Rabbit embryo at the compact morula stage. The nucleus (N), the Golgi complex (GK), mitochondria (M), lipid droplets (L), vesicles with a flocculent material (FV), the dense bodies (DB), residual bodies (RB). TEM, x7 200.

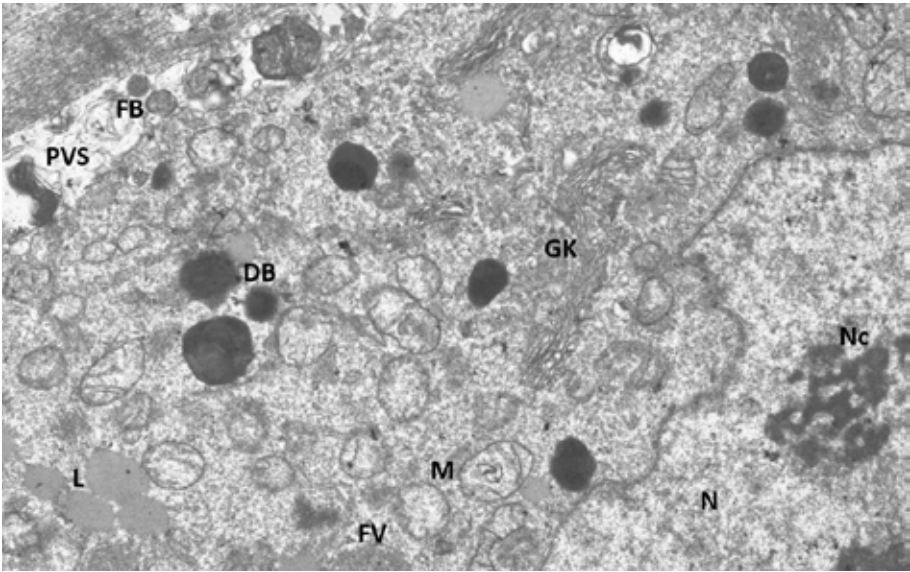


Figure 8.8 Rabbit embryo at the morula stage with reduced viability. The nucleus (N), nucleolus (Nc), the Golgi complex (GK), mitochondria (M), lipid droplets (L), vesicles with a flocculent material (FV), the dense bodies (DB), cell fragments (FB) in the perivitelline space (PVS). TEM, x7 200.

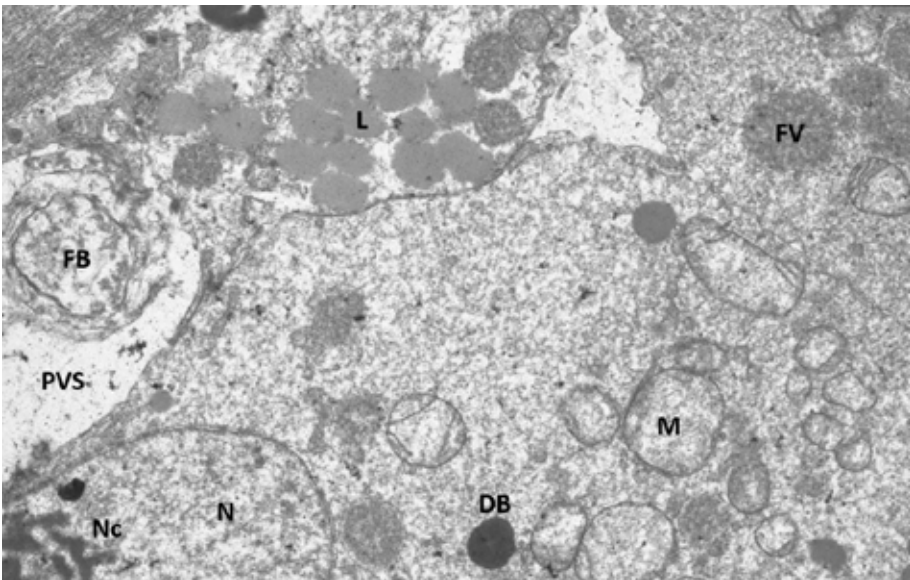


Figure 8.9 Rabbit embryo at the morula stage with reduced viability. The nucleus (N), nucleolus (Nc), mitochondria without cristae (M), lipid droplets (L), vesicles with a flocculent material (FV), the dense bodies (DB), large fragments of cells (FB) in the perivitelline space (PVS). TEM, x7 200.

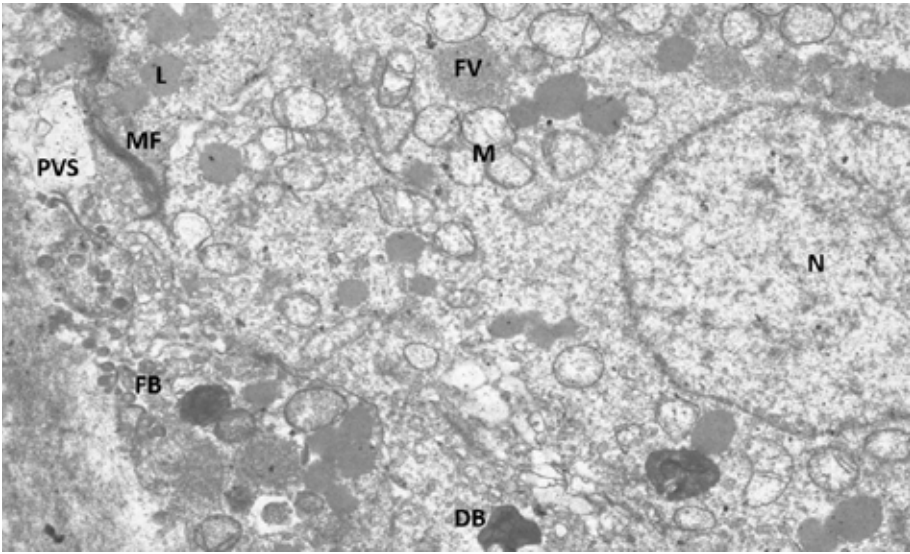


Figure 8.10 Rabbit embryo at the compact morula stage with reduced viability. Euchromatic nucleus (N), mitochondria without cristae (M), lipid droplets (L), vesicles with a flocculent material (FV), the dense bodies (DB), microfilaments of the tight junction *zonula adherens* (MF), large fragments of cells (FB) in the perivitelline space (PVS). TEM, x7 200.

9. Rabbit mezenchymal and amniotic stem cells ultrastructure

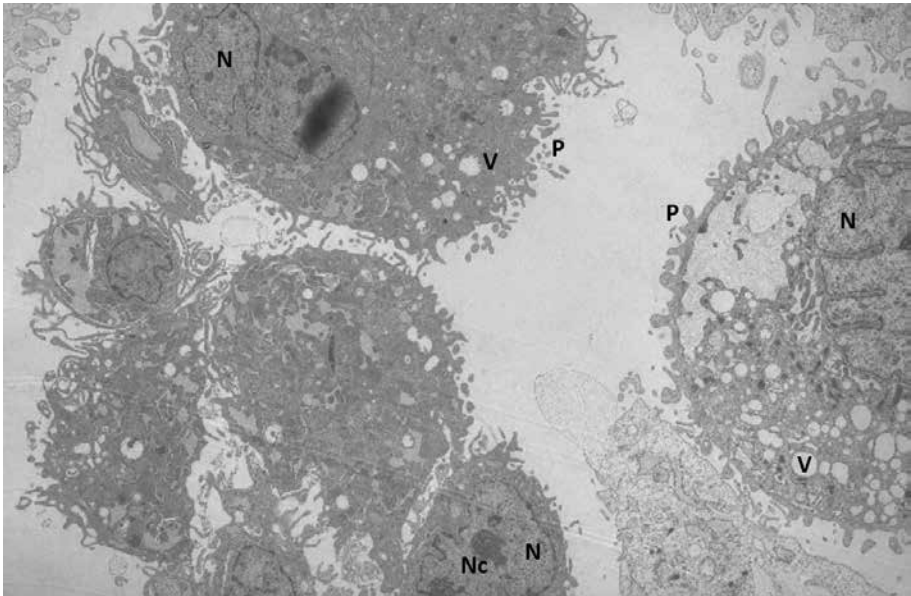


Figure 9.1 The group of rabbit mesenchymal stem cells. The nucleus of irregular shape (N) arranged eccentrically, numerous compact nucleoli (Nc) without segregation of nucleolar ribonucleoprotein components, small vacuoles (V), the thin pseudopodia (P), formed on the surface of the cytoplasm membrane. TEM, SCAN mode, x 1 900.

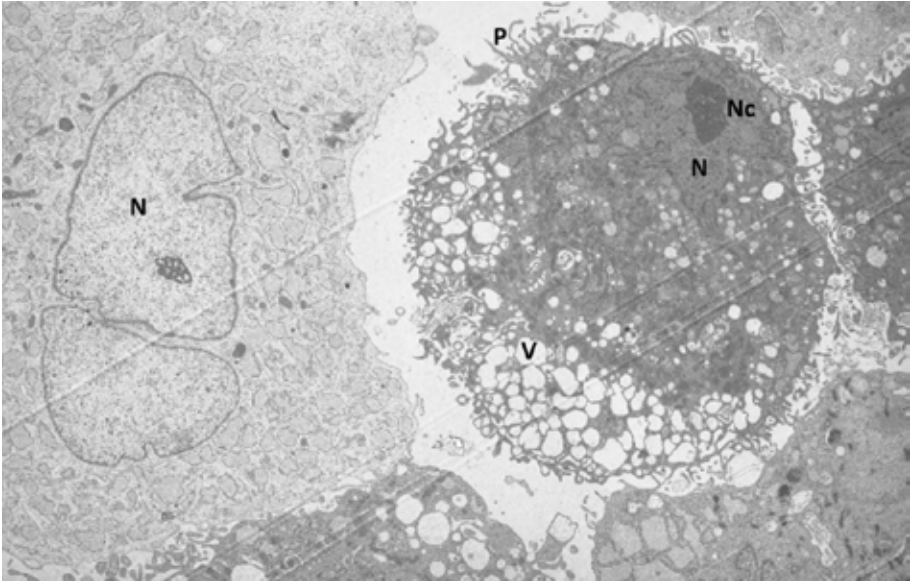


Figure 9.2 The rabbit mesenchymal stem cell. The nucleus of irregular shape (N) arranged eccentrically, the compact nucleolus (Nc) without segregation of nucleolar ribonucleoprotein components, numerous vacuoles (V), the thin pseudopodia (P), formed on the surface of the cytoplasm membrane. TEM, SCAN mode, x 1 900.

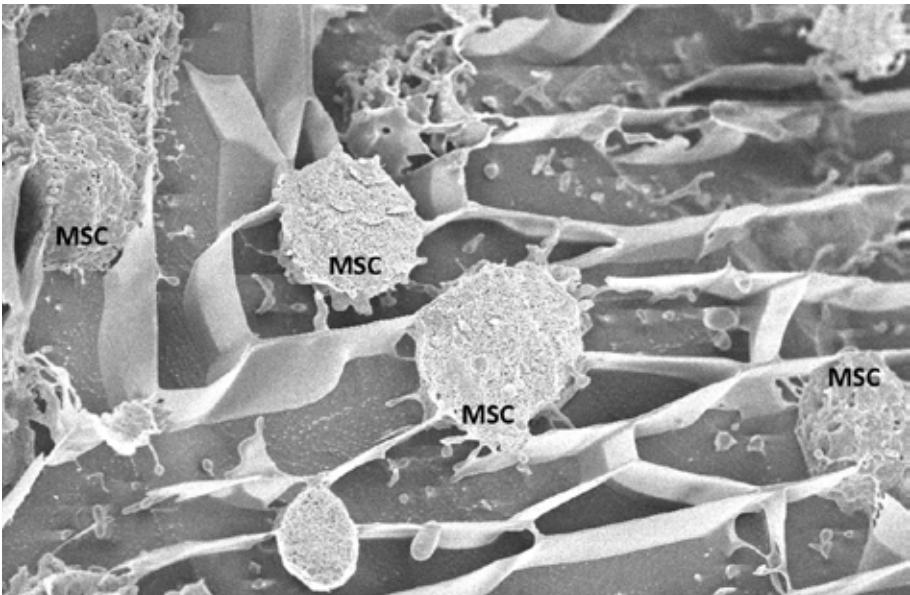


Figure 9.3 The group of entire and cross-sectioned mesenchymal stem cells (MSC) of a rabbit. Cryo-SEM, x 1 000.

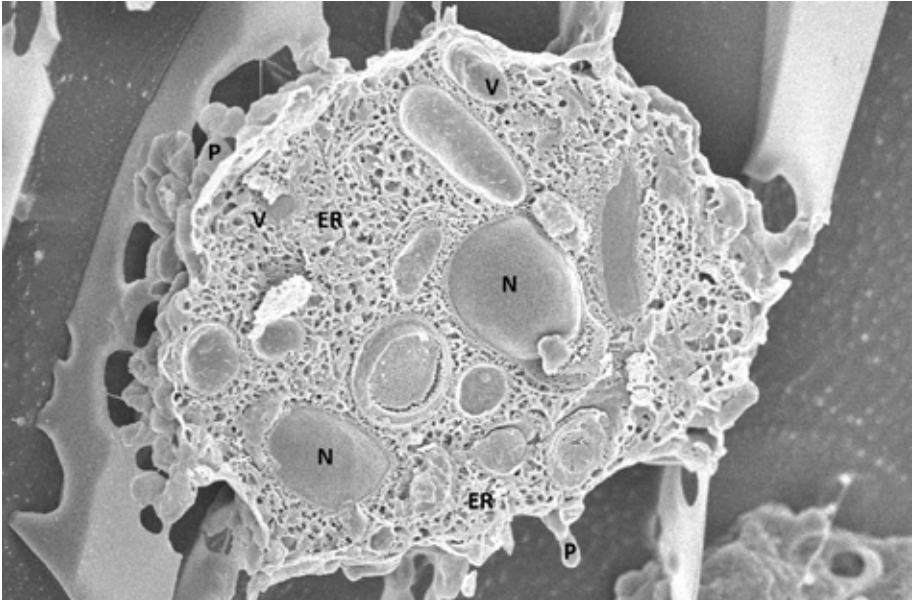


Figure 9.4 Cryosection across the middle of rabbit mesenchymal stem cell. Microarchitecture of cisterns and tubules of endoplasmic reticulum (ER) diffusely spread throughout the cell. In the centre there are fragments of the cross-refracted lobate nucleus (N). In the cytoplasm there are numerous vacuoles (V) of different sizes. On the cell surface there are differently sized projections of the cytoplasm in form of pseudopodia (P). Cryo-SEM, x 3 000.

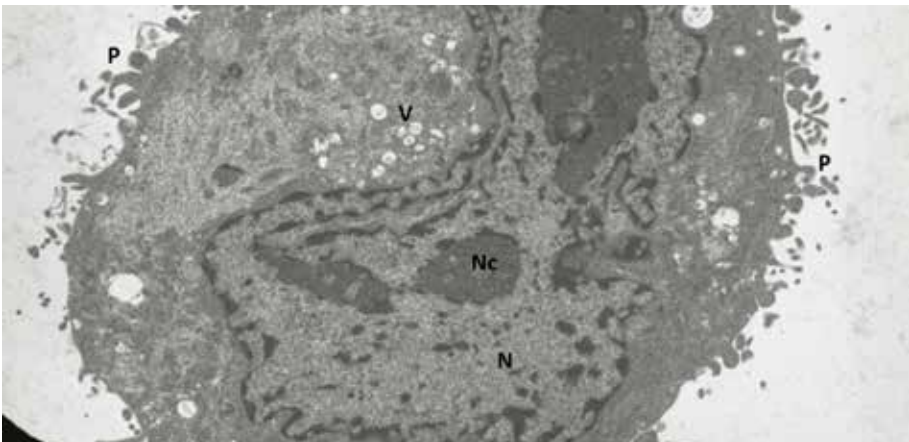


Figure 9.5 Rabbit amniotic stem cell. The nucleus (N) is kidney-shaped, eccentrically arranged; three large compact nucleoli (Nc) without segregation of nucleolar ribonucleoprotein components, small vacuoles (V), thin pseudopodia (P) formed on the surface of the cytoplasm membrane. TEM, x 5 800.

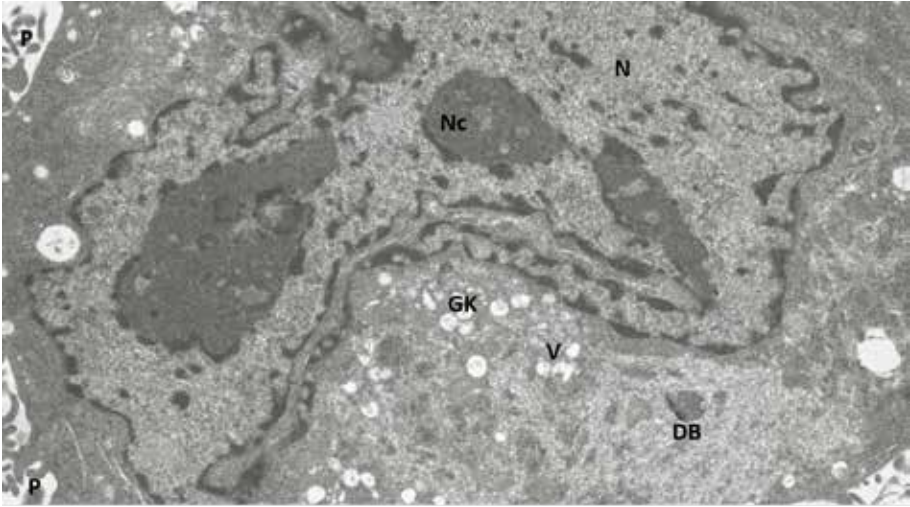


Figure 9.6 The image of an identical rabbit amniotic stem cell at a higher magnification. The nucleus (N), compact nucleoli (Nc) without segregation of nucleolar ribonucleoprotein components, Golgi complex (GK), vacuoles (V), the dense bodies (DB), pseudopodia (P). TEM, x 7 200.

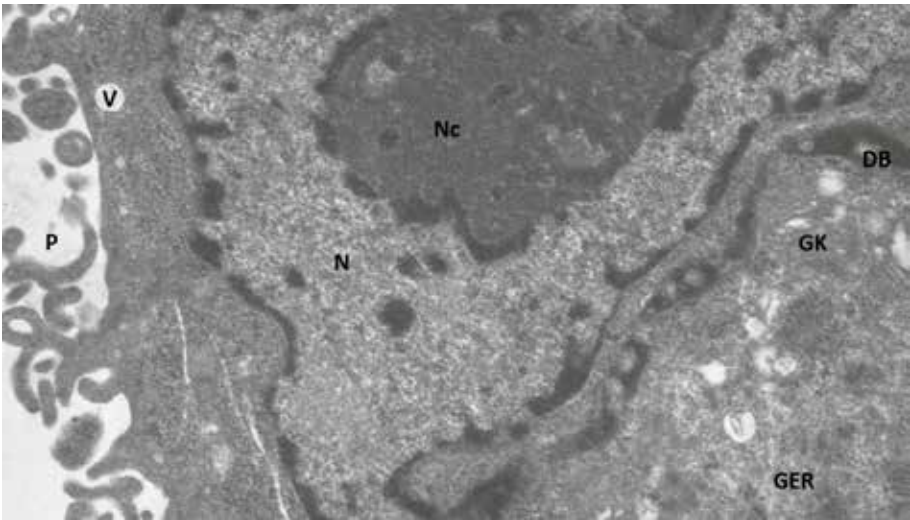


Figure 9.7 The image of an identical rabbit amniotic stem cell at a higher magnification. The nucleus (N), compact nucleoli (Nc) without segregation of nucleolar ribonucleoprotein components, Golgi complex (GK), granulated endoplasmic reticulum (GER), vacuoles (V), the dense bodies (DB), pseudopodia (P). TEM, x 14 000.

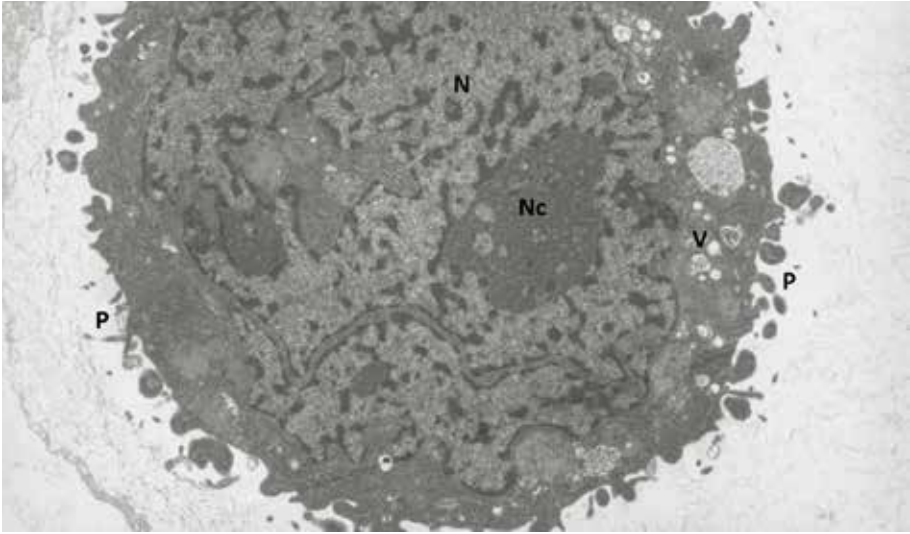


Figure 9.8 Rabbit amniotic stem cell. Large oval-shaped nucleus (N), centrally arranged; large compact nucleolus (Nc) without segregation of nucleolar ribonucleoprotein components, small vacuoles (V), thin pseudopodia (P), formed on the surface of the cytoplasm membrane. TEM, x 5 800.

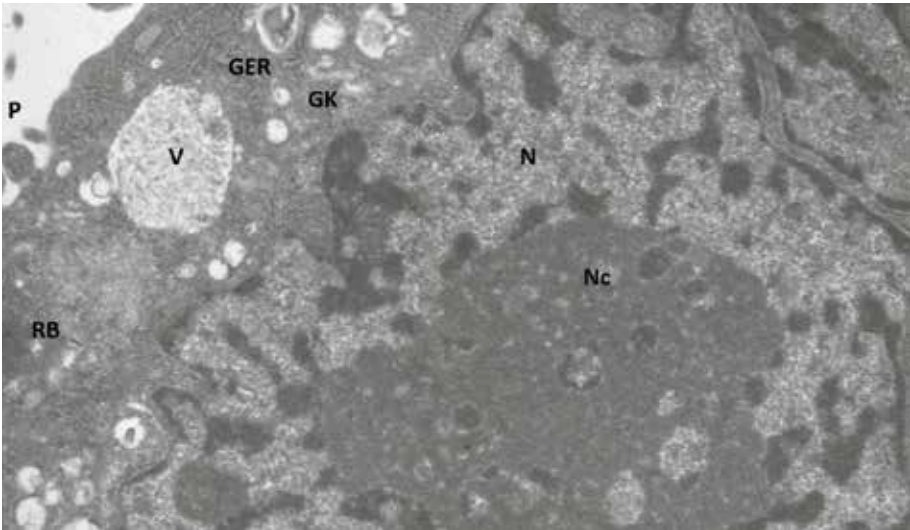


Figure 9.9 The image of an identical rabbit amniotic stem cell at a higher magnification. The nucleus (N) and its rugged surface, a large compact nucleolus (Nc) without segregation of nucleolar ribonucleoprotein components, Golgi complex (GK), granulated endoplasmic reticulum (GER), vacuoles (V), the residual body (RB), pseudopodia (P). TEM, x 14 000.

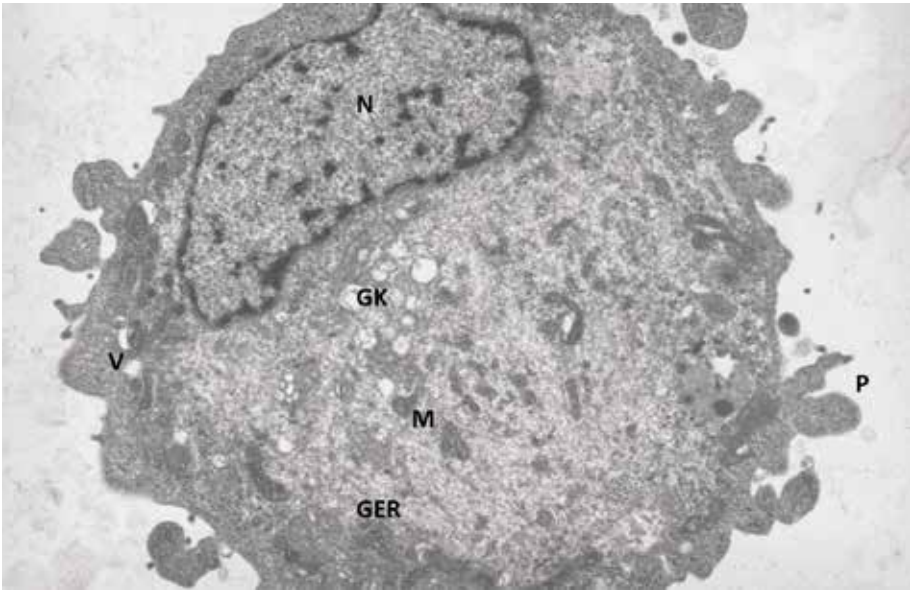


Figure 9.10 Rabbit amniotic stem cell. The kidney-shaped nucleus (N), eccentrically arranged, the Golgi complex (GK), granulated endoplasmic reticulum (GER), mitochondria (M), small vacuoles (V), pseudopodia (P) formed on the surface of the cytoplasm membrane. TEM, x 7 200.

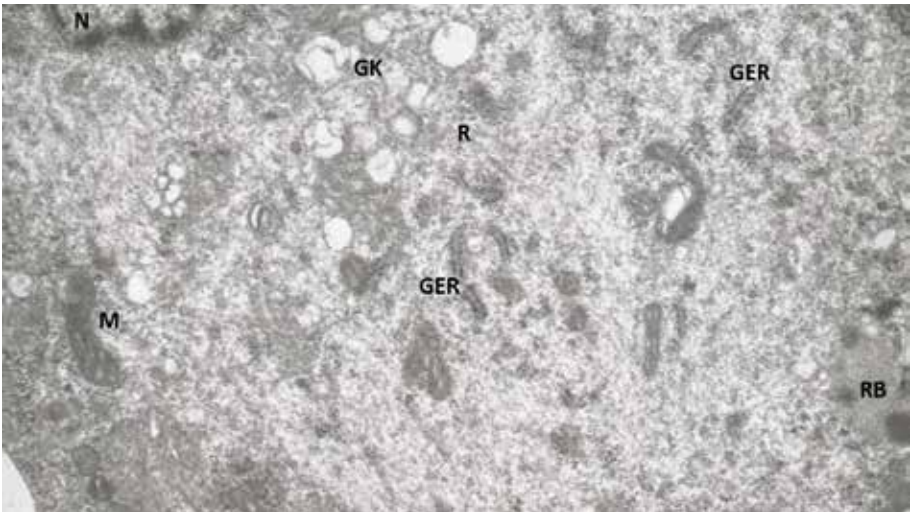


Figure 9.11 Image of an identical rabbit amniotic stem cell at a higher magnification. The nucleus (N), the Golgi complex (GK), granulated endoplasmic reticulum (GER), free polyribosomes (R), mitochondria (M), the residual body (RB). TEM, x 14 000.

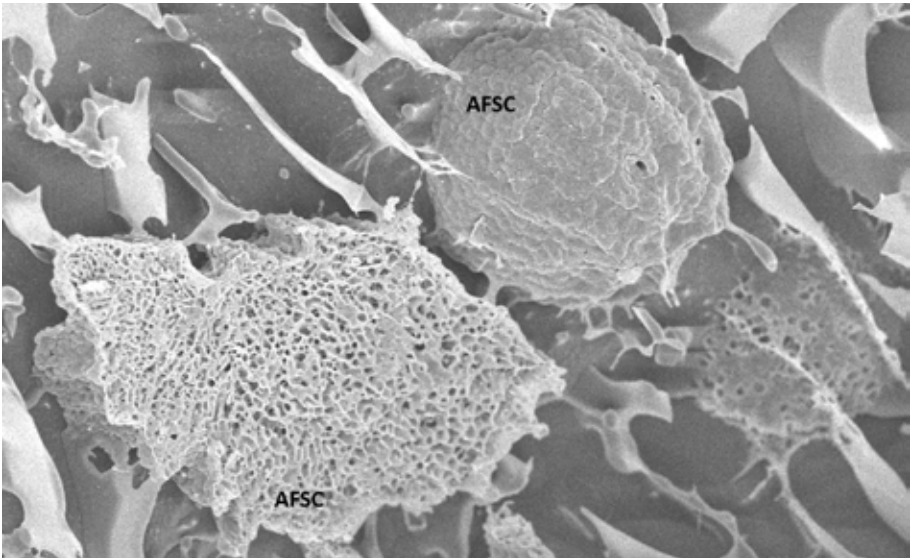


Figure 9.12 Image of entire and cross-sectioned rabbit amniotic stem cells (AFSC). Cryo-SEM, x 1 500.

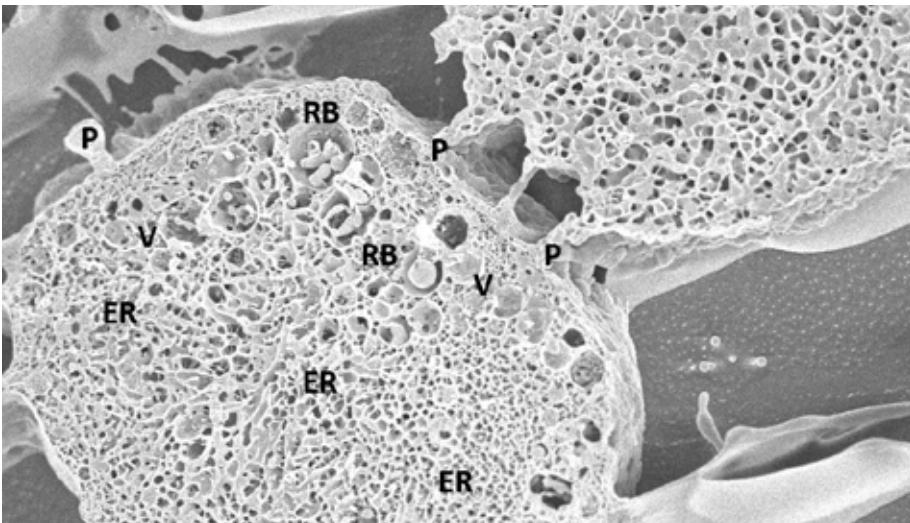


Figure 9.13 Cryosection across the middle of two rabbit AFSCs cells in close contact via pseudopodia (P). Microarchitecture of cisterns and tubules endoplasmic reticulum (ER) formed by the interconnected and diffusely distributed membranes throughout the cell. On the circuit, under the cytoplasm membrane there are numerous vacuoles (V), residual bodies (RB) of different sizes containing residues. On the surface of cells there are differently sized cytoplasmic projections in the form of pseudopodia (P). Cryo-SEM, x 2 000.

10. Ultrastructure of sperm phagocytosing rabbit cells

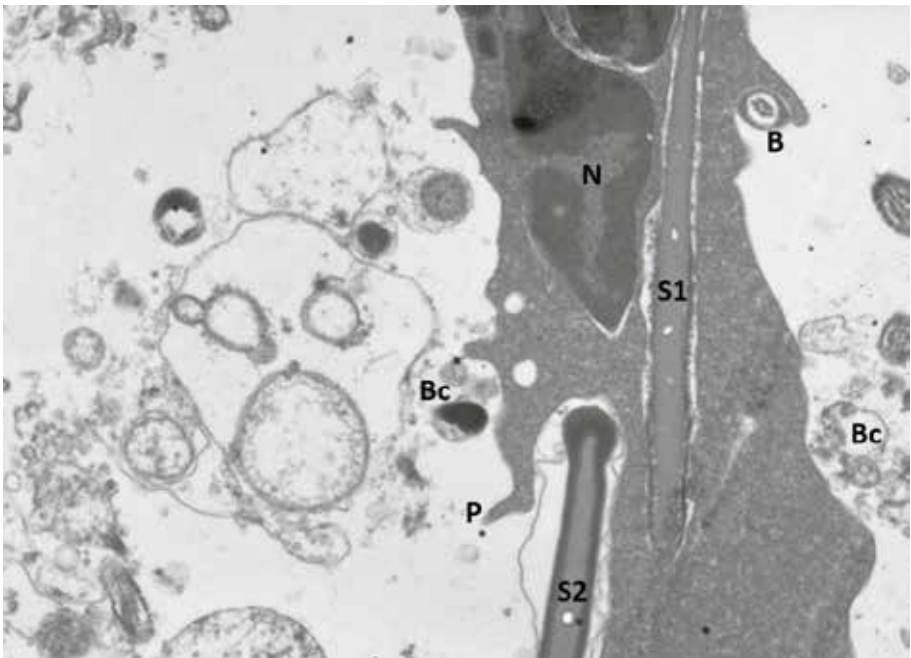


Figure 10.1 Macrophage in the rabbit ejaculate.

One phagocytosed sperm in the cytoplasm (S1), one sperm head (S2) on the surface, surrounded by the cytoplasmic projections (P) at the stage of absorption; in the upper part of the cytoplasm there is a macrophage nucleus (N) and the cross-section of a flagellum (B) of the other absorbed sperm, cell particles (Bc) in the vicinity of a macrophage, probably remnants of the digestive process. TEM, x 7 200.

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Summary

Short generation interval, characterized by a period from the birth up to the ability to reproduce and create further progeny, and high fertility are useful properties which predetermine the use of rabbits for experimental purposes within the biotechnological and biomedical research. This fact was the reason that the experimental research, the results of which are presented in the given publication, has been implemented on a rabbit model.

The publication is divided into ten chapters. The first chapter provides knowledge about reproductive (oocyte, sperm and embryo) cells. It includes a description of individual developmental stages of the egg from the oocyte to the blastocyst in order to create their morphological characteristics, as well as the description of the structure and function of sperm.

The second chapter focuses on stem cells. In view of the expanding use of stem cell therapy, not only in the field of hematology and oncology but also in the treatment of cardiac, neurological, autoimmune and other diseases, this chapter is concentrated on the detailed description of individual types of stem cells and their role in the organism.

The third chapter is devoted to sperm phagocytic cells. It contains their characterization, basic classification as well as a description of their significance in the reproductive system of males. In addition to theoretical knowledge, all three chapters are richly supplied by a properly selected pictures.

The fourth chapter focuses on the description of the basic (classical) and modern methods of the quality assessment of gametes and

stem cells, which are currently intensively used in research and practice.

Next, the fifth chapter provides methodology for the preparation and processing of samples of biological material for evaluation by electron microscopy. Part of the chapter is a detailed description of the ultrastructure of cell organelles.

The last four chapters (sixth to tenth) include the picture documentation, which was obtained on the basis of ultrastructural analysis of oocytes, sperm, embryos, amniotic and mesenchymal stem cells, as well as spermphagocytosing cells of a rabbit. Individual images are accompanied by detail description of organelles, which are located in the cells.

The publication is useful for students of colleges and universities, post-graduate students and for workers dealing in areas related to reproduction and animal biotechnology.

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