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Review

The morphological and functional ultrastructure of cells in pre-implantation embryos

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Abstract. Cells of pre-implantation embryos are equipped with many morphological and functional systems through which they can synthesize specific proteins and effectively ensure the protection of early embryonic development. Here we present evidence for the existence of these systems in morphologically normal and abnormal bovine blastocyst stage embryos *in vivo* at the ultrastructural and actin cytoskeleton levels. The appearance of organelles in the trophectoderm and inner cell mass (ICM) cells, responsible for their synthetic activities and their role in the development of early bovine embryos are described. We point out the importance of endocytic processes and the participation of extracellular vesicles in the formation of intercellular contacts and homeostasis of the embryo microenvironment. Several changes in the ultrastructural morphology of embryos produced by different methods (ICSI, parthenogenetic AC/DC electrical activation, IVF with separated sperm) and freezing/thawed embryos are described. We also show alterations occurred in the organelles after viral contamination of embryos with BHV-1 and BVDV viruses, and in embryos from over-conditioned cows. Recorded changes in organelles and appearance of cellular autophagic structures (vesicles, multivesicular bodies and autophagolysosomes) may negatively affect embryo metabolism and lead to the emergence of pathological processes in trophectoderm and ICM cells of preimplantation embryos.

Key words: Bovine — Embryo — Endocytosis — Ultrastructure — Cytoskeleton — Extracellular vesicle

Abbreviations: ICM, inner cell mass; IVP, *in vitro* production; PvS, perivitelline space; ER, endoplasmic reticulum.

Introduction

In eukaryotic cells, Cheville (1994) differentiates six different cooperating systems: surface, nucleus-nucleolus-ribosomes-rough endoplasmic reticulum (ER) and Golgi complex, vacuolar system, smooth ER, mitochondria and cytoskeleton. These systems, according to their morphological-functional meaning, characterize their functional capability. This type of classification of cell structures and their functions was further developed in the study of Zibrín et al. (2005) on Sertoli cells of mammals. We applied this classification in our study on bovine preimplantation embryos.

The surface of each cell is formed by a cell membrane, which ensures the reception of electrical and chemical

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signals and the transport of substances into and out of the cell. Electrical signal means the transport of a substance through the membrane referred to as secondary active transport, or co-transport (Dubyak 2004). To transfer molecules across the membrane, the co-transport does not use ATP directly, but the necessary energy comes from the electrochemical gradient created by pumping ions out of the cell. Specifically, this can be done from the higher extracellular Na^+ concentration (chemical potential) compared to the intracellular Na^+ concentration and from the transmembrane potential (electrical potential), which is negative inside the cell and, therefore, attracts Na^+ ions into the cell. We may assume that the nutrients can also be absorbed by the trophoblast membrane by co-transport. The cell surface creates various specialized structures of the cell, such as microvilli and motile cilia, intercellular junctions and basal structures.

The intensity of the appearance of microvilli on the surface of cells facing the perivitelline space (PvS) has a fluctuating character in different embryonic stages of development. At the zygote stage, the occurrence of microvilli is intense, while at other stages (2-, 8- and 16-cell stage) their occurrence decreases, and then increases again at the morula and blastocyst stages (Plante and King 1994). Microvilli are abundant in all mammalian blastocysts studied to date, suggesting that they likely play an important role in development (Mohr and Trounson 1981).

Nucleus-nucleolus-ribosomes-rough ER and the Golgi complex form organelles that decode the genome and produce and secrete many specific proteins – glycoproteins.

The smooth ER is the site of lipid synthesis, and, at the same time, it is the cell detoxification system.

Mitochondria are organelles in which energy transformation takes place. They produce molecules from which other systems use energy. A marked enhance in embryonic metabolism occurs at the stage after the activation of the embryonic genome at the time of blastocyst expansion (Leese 1995). At this stage, elongated mitochondria are capable of ATP production. Inhibition of oxidative phosphorylation in bovine blastocysts, thus, can have a negative effect on their development and quality (Thompson et al. 2000).

The vacuolar system ensures the transport of substances between the organelles of the cell and within the secretion of the cell, as well as intracellular digestion through lysosomes.

The cytoskeleton consists of microtubules, microfilaments, intermediate fibers and includes centrioles. The cytoskeleton forms the structural support of the cell. It serves for intracellular transport, movement of organelles and intracellular inclusions inside the cell.

On the day 6 post-insemination (6 dpi), the bovine embryo reaches the blastocyst stage with the formation of two distinct cell lines: inner cell mass (ICM) and trophectoderm cells (Van Soom et al. 1997). Compaction of the early bovine embryo occurs between the 32–64 cell stage of development (Van Soom et al. 1997) and the first formation of ICM cells has appeared as early as the 10–32 cell stage in bovine embryos (Van Soom et al. 1996). From a developmental point of view, the cells of the ICM are the basis for the future fetus, while cells of the trophectoderm form the trophic and nutritional systems of the fetal envelope. In the cytoplasm of these cells, like eukaryotic cells, morphological structures (morphological-functional systems) characterized by their metabolic activity are gradually formed in the preimplantation period. The success of development depends significantly on their morphological quality (Van Soom et al. 1992).

The aim of this study was to describe and compare the occurrence and ultrastructure of functional systems according to the criteria of Cheville (1994) and Zibrín et al. (2005) in the cells of morphologically normal and abnormal bovine early embryos during their preimplantation period.

Experimental aspects

Embryos at the blastocyst stage (*in vivo*, 7 dpi), flushed from Holstein-Friesian cows, were used in the study. Their quality was assessed under a stereomicroscope. We classified the embryos as normal or abnormal after morphological evaluation using a light microscope based on presence or absence of cellular fragments in the PvS, state and the shape of the *zona pellucida* and PvS.

Morphologically normal embryos manifested intact *zona pellucida* and not more than 5 fragments in the PvS, which was not expanded. Morphologically abnormal embryos were characterized by either disrupted *zona pellucida* or more than 5 fragments in the PvS, which was expanded. At the ultrastructural level, normal blastocysts were characterized by a narrow PvS, continuous coverage of numerous stacked microvilli on the plasma membrane and well-defined cell-to-cell junctions. The abnormal blastocyst showed further shrinkage with the expansion in PvS, damage to the microvilli and accumulation of cellular debris in the PvS.

For ultrastructural analysis, embryos were processed using routine approach and evaluated with a JEM 100 CX II transmission electron microscope (JEOL) at an accelerating voltage of 80 kV.

To label actin filaments of the cytoskeleton, blastocysts were incubated in a phalloidin-TRITC solution (Actin Cytoskeleton and Focal Adhesion Staining kit, Chemicon Int., CA, USA) for 45 min. Subsequently, the embryos were washed in a phosphate buffer, placed on a coverslip, covered with the Vectashield mounting medium with fluorescent dye DAPI (Vector Laboratories, CA, USA), mounted on a glass slide and prepared for fluorescence analysis using confocal microscopy.

Table 1. Occurrence of structural systems in cells of normal (grade 1) and abnormal (grade 3) *in vivo*-derived cow embryos

Cell structures	Morula	Blastocyst	Morula	Blastocyst
	good quality (grade 1)		poor quality (grade 3)	
Surface/microvilli (TE)	+++	+++	+	+
Nucleus/nucleolus (FG)	+++	+++	++	+
Golgi complexes	+++	+++	+	+
Rough ER	++	++	+	++
Smooth ER	+++	+++	++	+
Mitochondria	+++/E/H	+++/E/H	+++/E/H	+++/D
Vacuoles	++	+	++	+++
Cytoskeleton/IF(TE)	+	+	+	+
Lipids	+	+	++	+++
MVT/AV	+	+	++	+++

TE, trophectoderm; FG, fibrillo-granular; ER, endoplasmic reticulum; IF, intermediate filaments; MVT, multi-vesicular bodies; AV, autophagic vacuoles; E, elongated; H, hooded; D, damaged. Intensity of occurrence: +++ high, ++ moderate, + low.

Structural systems of cells of normal early bovine embryos

Bovine blastocysts, evaluated with a light microscope as a grade 1, have symmetrical and largely intact blastomeres, approximately the same size and shape, connected by intercellular junctions. They are formed by flat cells of the trophoblast, regularly arranged in one thin surface layer like the epithelium, surrounding a cluster of cells of the ICM and a cavity – blastocoel. Differentiated structures are well recognizable when evaluating the blastocyst in its native state (Fig. 1).

The different occurrence of structural systems of cells in morphologically normal bovine morulae and blastocysts *in vivo* is presented in Table 1.

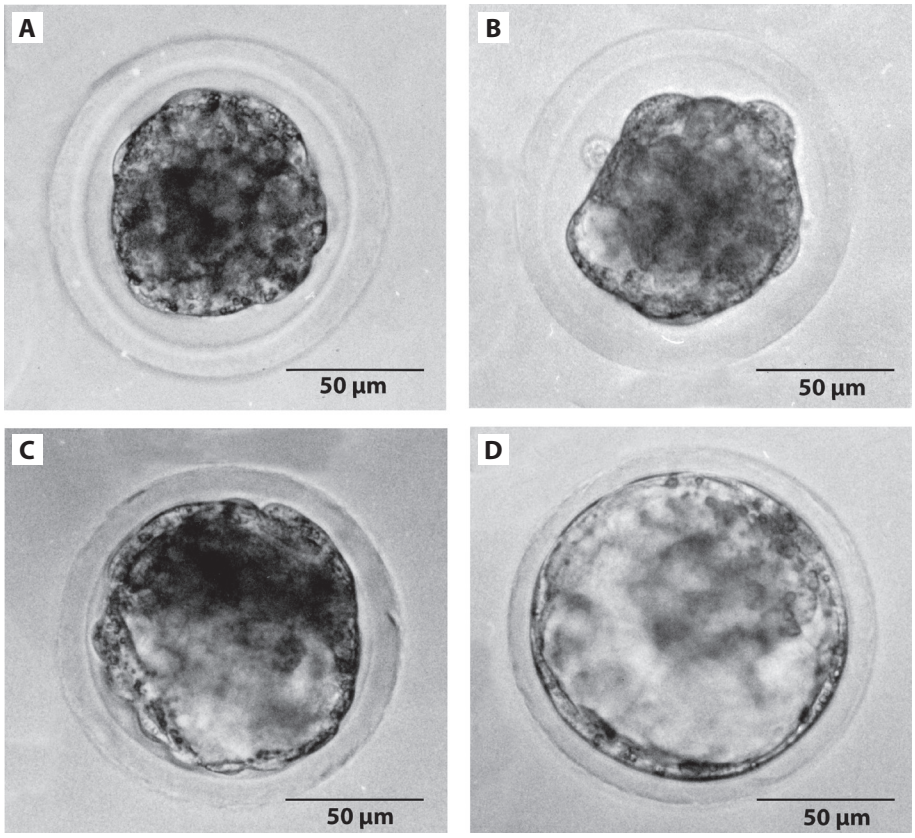

Figure 1. Morphology of the normal bovine blastocyst (grade 1). Different stages of *in vivo* bovine embryos (all classified as a grade 1). **A.** Morula – 5 dpi (days post-insemination). **B.** Early blastocyst – 6 dpi. **C.** Blastocyst – 7 dpi. **D.** Expanded blastocyst – 8 dpi. Native preparation, ×400. Photo: in Pivko et al. (2013), pp. 122-123.

The cell surface

Trophectoderm cells have a distinctly polar character, and their free surface is fragmented, with well-developed microvilli oriented into the PvS. High microvilli at the apical end of flat trophoblast cells are numerous and oriented into the PvS (Fig. 2A). In mouse oocytes, actin filaments from the cortical layer penetrating the internal microvilli structure (Karasiewicz and Soltyňska 1985). At the base of the microvilli, we observe pinocytotic vesicles with a sparse content, directed to the interior of the cytoplasm.

Numerous developed microvilli are a prerequisite for more effective blastocyst development (Linares and Plöen 1981). Microvilli are reduced in the regions of blastomere

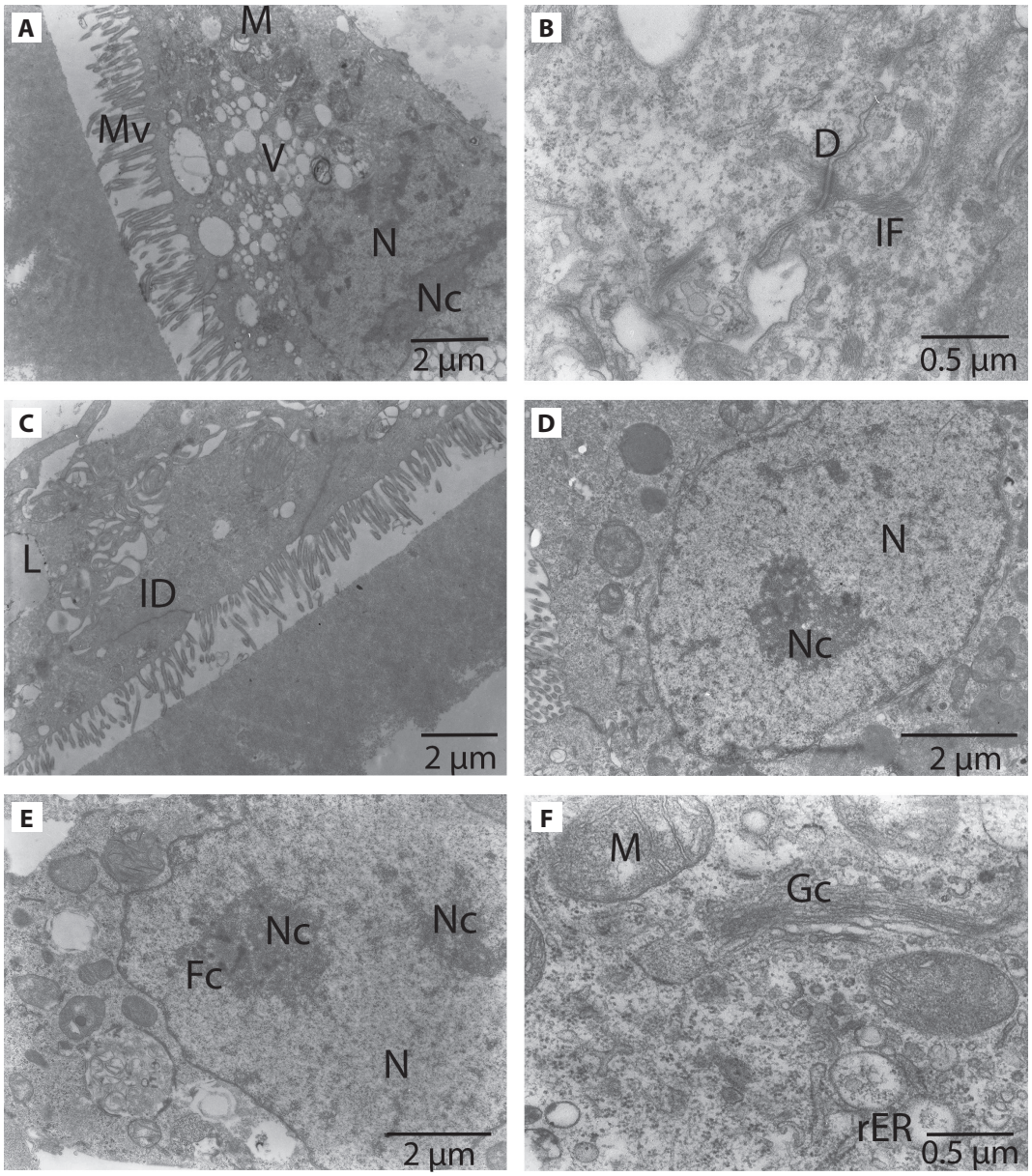

Figure 2. Ultrastructure of the morphologically normal bovine blastocyst (grade 1). **A.** A rim of microvilli (Mv) on the apical surface of flat trophectoderm cells. In the cytoplasm, vesicles (V), mitochondria with cristae (M), nucleus (N) and nucleolus (Nc), ×5000. **B.** Desmosomes (D), intermediate filaments (IF), ×20 000. **C.** Prominent interdigitations (ID) of trophectoderm cells on their basal surface. ×5000. L, lipid droplet. **D.** A large oval nucleus (N) of the heterochromatin type and a compact nucleolus (Nc) formed by fibrillary and granular components in the cytoplasm of trophectoderm cells, ×6700. **E.** An oval nucleus (N) and two differently sized compact nucleoli (Nc) with a partially uniform distribution of ribonucleic components and a fibrillary centre (Fc) are visible in the cytoplasm of the inner cell mass (ICM) cells, ×6700. **F.** Rough endoplasmic reticulum (rER), Golgi complex (Gc) and mitochondria (M), ×20 000.

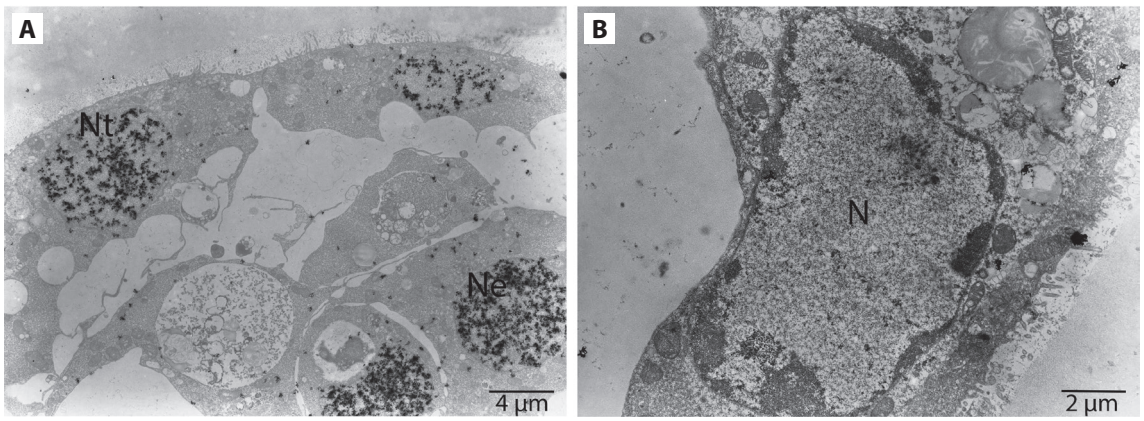

Figure 3. Ultrastructural autoradiography of the morphologically normal bovine blastocyst. **A.** Autoradiographic localization of [5-^{3}H] uridine (dark grains) incorporated into the nucleus. More intensive RNA synthesis in the nuclei of the ICM (Ne) than in the nuclei of the trophectoderm cells (Nt). **B.** In the centre of the trophectoderm cell, a large nucleus (N) without RNA synthetic activity.

apposition and multiplied on the surface of cells facing the PvS and, thus, increase the absorption surface and are a means of supporting the development of bovine embryos (Mohr and Trounson 1981). Trophectoderm cells on the basal surface do not form microvilli. The cell membrane forms various intercellular junctions. Junctional complexes on the lateral sides of the cells are well developed and include a series of desmosomes (Fig. 2B), from which intermediate filaments depart into the cytoplasm. The *zonulae occludentes*, *zonulae adherens* and desmosomes are commonly occurred (Prather and First 1993). The membrane of trophectoderm cells on the lateral and basal surface often creates cytoplasmic protrusions that fit into each other in the form of interdigitations (Fig. 2C) and performs the strengthening functions of the embryo. The trophectoderm cells on the basal surface begin to form a basement membrane separating the trophoblast from the endoderm cells. The ICM cells do not show any polarity. Their intercellular connections are few, what results in numerous extensions between cells. Neither *zonulae adherens* nor desmosomes were observed that ensure contact between ICM cells. The cell membrane between the cells of the ICM forms only gap junctions.

Proteosynthetic structures

Successful embryonic development is preceded by intense synthetic RNA activity of the nucleolus (Motlík et al. 1978) and glycoprotein synthesis during the maturation of the oocyte (Pivko et al. 1982). The proteosynthetic apparatus is relatively well developed, especially in embryos of higher developmental stages with a functional embryonic genome, which is species-specifically differentiated (Kopečný and Niemann 1993).

In the cytoplasm of trophectoderm cells, large oval nuclei contain clumps of chromatin distributed throughout the nucleoplasm and several nucleoli. Large compact nucleoli are formed by fibrillary and granular components (Fig. 2D). A similar image of an oval nucleus and two compact nucleoli of different sizes with a partially uniform distribution of ribonucleic components and a fibrillary center are observed in the cytoplasm of ICM cells (Fig. 2E). In the cytoplasm, there are numerous free ribosomes and polyribosomes, rough ER and Golgi complexes with numerous vesicles, distributed around lipid vesicles (Fig. 2F). At early stages after fertilization, Golgi complex aggregations are located near the nucleus and participate in the formation of cortical granules, but until the blastocyst they are not essential for early embryonic development (Payne and Schatten 2002). Structural dynamics of Golgi complexes during embryonic cell cycles resembles those of somatic cell cycles. Rough ER is more numerous while smooth ER decreases (Maddox-Hyttel and Boerjan 2002).

During the autoradiographic study of the incorporation of labelled uridine, we found a more intensive RNA synthesis in the ICM nuclei of morphologically normal bovine blastocysts than in the trophectoderm nuclei, which may express quantitatively different production of specific proteins (Fig. 3A). In some nuclei, chromatin was concentrated in large peripheral blocks but labeling was absent (Fig. 3B). Similar chromatin patterns were observed in the earlier study by Pivko et al. (1986).

These functional systems ensure the synthesis of specific substances of a humoral nature like hormones, which are responsible for the application of endocrine mechanisms ensuring undisturbed preimplantation and post-implantation embryonic development. An early bovine embryo around the 15th–17th day of pregnancy secretes interferon-τ, which

changes the production of prostaglandins and, thus, prevents luteolysis. In terms of functionality, the trophectoderm cells are specialized for many functions, mainly the ability to absorb nutrients, selective transport, active metabolism with hormone production and resistance to maternal immunological attacks (Peter et al. 2017).

Fertility rates in animals are limited by early embryonic losses, and in cows the highest incidence of losses was found between days 8 and 18 after ovulation (Rodgers 2001; Diskin et al. 2006). The rapid expansion of a functional and intact trophoblast plays an important role in this direction, when it provides the nutrition of the embryo through the pinocytosis of nutrients and the stimulation of the *corpus luteum* through the synthesis of the signal product – interferon-τ (Roberts 1989; Dorniak et al. 2013). However, this embryo-maternal information must start working before the 16th day after ovulation. The signaling product – interferon-τ is a specific protein synthesized by trophectoderm cells. The mechanism of its action is not fully understood, but it is believed to stimulate the activity of the *corpus luteum* by altering the production of prostaglandins and preventing luteolysis. In addition to interferon-τ, non-implanted blastocysts of cows, sheep, pigs and horses are capable of estrogen synthesis (Perry et al. 1976). In comparison, pig blastocysts produce these estrogens as early as day 12, thereby inhibiting uterine **prostaglandin** production, which causes regression of the *corpus luteum* in the absence of embryos. On the other hand, in sheep, anti-luteolytic activity may be exerted *via* a **glycoprotein** (Findlay et al. 1979).

Smooth ER

In metaphase II oocytes, smooth ER forms clusters of spherical form located in the peripheral zone below the oolemma (Maddox-Hyttel and Boerjan 2002). In the cells of early embryos, the smooth ER is few and consists of a system of anastomosing tubules and vesicles bounded by a smooth membrane. Flat cisternae occur rarely in trophectoderm and ICM cells. Early embryos contain little amount of ER, but annular lamellae, as precursors of smooth ER, are often present (Maddox-Hyttel and Boerjan 2002). Functionally, the smooth ER is the site of steroid synthesis in eukaryotic cells (Christensen 1975) and at the same time is an important detoxification system of the cell. Biosynthesis of prostaglandin synthase and enzymes responsible for the synthesis of progesterone, testosterone and possibly estradiol has been demonstrated in bovine *in vivo* blastocysts collected at days 13, 15 and 16 (Shemesh et al. 1979), which corresponds to the elongated blastocyst stage.

Mitochondria

In oocytes and earlier embryonal stages, mitochondria of the hooded form (hood-like) predominate. They are immature mitochondria that have few peripherally located cristae and are typical of early stages of embryonic division (Brackett et al. 1980; Enders and Schlafke 1981). In morulaes and blastocysts, electron-lucent elongated mitochondria with prominent cristae already predominate in close contact with lipid droplets. Complete elongation of mitochondria in bovine blastocysts is completed first in the cells of the trophectoderm and only later in the cells of the embryoblast (Hayashi et al. 2021). During *in vitro* maturation of bovine oocytes, peripheral mitochondria showed a spatial distribution, when they are translocated from the peripheral zone throughout the ooplasm and end up localized centrally (Hyttel et al. 1986). In porcine blastocysts, this translocation of mitochondria is mediated by microtubules and is homogeneously distributed in the trophoblast and embryoblast cells (Sun et al. 2001).

Cellular and extracellular vesicles

Small and large vesicles with different contents of morphological structures and lysosome-like bodies are found in the cytoplasm of cells of early embryos. These so-called cytosomes represent a very dynamic system that undergoes morphological and functional changes during the pre-implantation and post-implantation development of the embryo. The system of vesicles in the trophectoderm cells is well developed at the early stage of blastocyst development in conjunction with a prominent nucleus/nucleolar apparatus, which is related to their secretory activity, especially the production of specific proteins and blastocoel fluid. In the trophectoderm cells, numerous vesicles around the nucleus and large compact nucleoli without obvious segregation of nucleolar components are often observed (Fig. 4A).

Cytoplasmic vesicles have been described in bovine embryos obtained both *in vivo* (Linares and Plöen 1981) and *in vitro* (Shamsuddin and Rodriguez-Martinez 1994), as well as in primate embryos (Enders and Schlafke 1981). Together with morphologically and functionally similar cell structures, the vesicular system also consists of lysosomes and lysosome-like bodies (Fig. 4B). Lysosomes are also well developed serving for the enzymatic degradation of substances of intracellular and extracellular origin and the elimination of apoptosis (Hardy 1997). The cytoplasmic vesicles in contact with the plasma membrane are observed in the cells. Such cell cytoplasmic vesicles and especially extracellular vesicles in the intercellular microenvironment have been described in many studies as carriers of signaling products including proteins, lipids and small RNAs (Choi et al. 2013; Phinney et al. 2015; Maas et al. 2016). It has been found that human trophoblastic extracellular vesicles may play a key role in the maternal-placental-fetal communication (Ouyang et al. 2016).

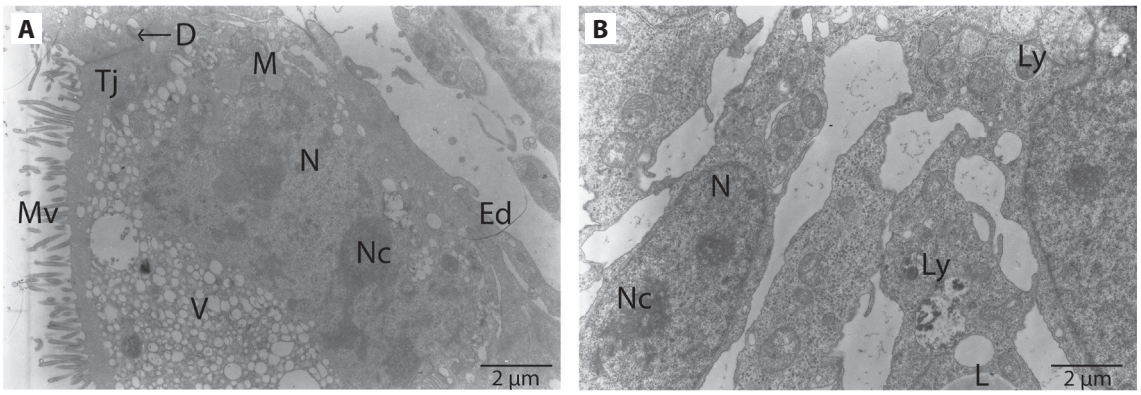

Figure 4. Ultrastructure of the vesicular system of the morphologically normal bovine blastocyst. **A.** Connection of two trophectoderm cells laterally by a tight junction (TJ) and a desmosome (D). Microvilli (Mv), numerous small and large vesicles (V) in the cytoplasm, mitochondria with cristae (M), nucleus (N) and nucleolus (Nc), cytoplasmic protrusions of the endoderm (Ed), ×5000. **B.** Numerous lysosome-like bodies (Ly) in ICM cells, nucleus (N) with nucleolus (Nc) and lipid droplet (L), ×5000.

Extracellular vesicles also contain the tetraspanin proteins, which are mostly used as markers in studying the biogenesis of extracellular vesicles in cells under physiological or pathological conditions (Jankovičová et al. 2020). The studies by Jankovicova et al. (2016, 2019) provided evidence for the involvement of the tetraspanin web proteins, CD9 and CD81, in the physiology of extracellular vesicles in mouse sperm, in oocytes and embryos of cows and pigs. They described the involvement of tetraspanins from extracellular vesicles in cell-to-cell contact and communication between cells of the mammalian reproductive system and their functions in gamete maturation, fertilization and embryo development.

The microenvironment, in which mouse embryoblast cells were located, is made up by extracellular vesicles produced by ICM cells (Cruz et al. 2018). It is assumed that extracellular vesicles can regulate specific intracellular target cell signaling and function through exosomes and microvesicles, which differ in size and content, depending on their biogenesis processes and biophysical properties (Witwer et al. 2013).

According to Cruz et al. (2018), exosomes are small homogenous particles with a diameter of 40 to 100 nm originating from a renewed, by fusion of lipids and proteins removed through endocytosis, cell membrane. The exosomes are intra-luminal vesicles located inside multivesicular bodies, which can fuse with the plasma membrane and release them into the extracellular environment as exosomes, or they can be directed for degradation *via* lysosomes (Fig. 5). In contrast, microvesicles are a more heterogeneous population ranging from 50 to 1000 nm in diameter and are produced by direct budding from the plasma membrane (Mathivanan et al. 2010; Raposo and Stoorvogel 2013; Kowal et al. 2016). The following reports (Scita and Di Fiore 2010; Dobrowolski and De Robertis 2011) are evidence that extracellular vesicles and their synthesized products probably play an important role in the regulation of intracellular signaling as modulators of the microenvironment.

Extracellular vesicles are beneficial for oocyte development processes shortly after fertilization and for subsequent embryo development in the pre- and post-implantation period. Thus, Pavani et al. (2020a) found that extracellular vesicles are secreted into the culture medium of embryos and, as such, represent a source of novel biomarkers to identify cell and embryo quality. Small amounts of bovine embryo-modified medium for extracellular vesicles separation were obtained by differential ultracentrifugation, OptiPrepTM density gradient centrifugation (ODG), and size exclusion chromatography. The authors found that ODG centrifugation is the preferred method to identify extracellular vesicles-enriched components and to improve our understanding of extracellular vesicles function in embryo quality and development. Two types of electron-lucent vesicles (heterogeneous and striated) were observed, which are probably the precursors of lipid droplets (Pavani et al. 2020a).

The mature bovine oocyte does not obviously have a yolk, but even before fertilization it can absorb nutrients through receptor-mediated endocytosis, indicating active energy utilization or storage (Pavani et al. 2020b). Also, Mellisho et al. (2017) demonstrated in their work that bovine blastocysts secrete microvesicles and exosomes into the culture medium. Electron microscopy revealed that microvesicles and exosomes are rounded in shape, enclosed by a lipid bilayer and range from 30 to 385 nm in diameter. The authors proved that the concentration of vesicles is significantly higher in IVF (*in vitro* fertilization) blastocysts from days 9 to 11 of development (6.7×10^8 particles/ml) compared to parthenogenetic blastocysts (4.7×10^8 particles/ml). Similarly, the profile (concentration

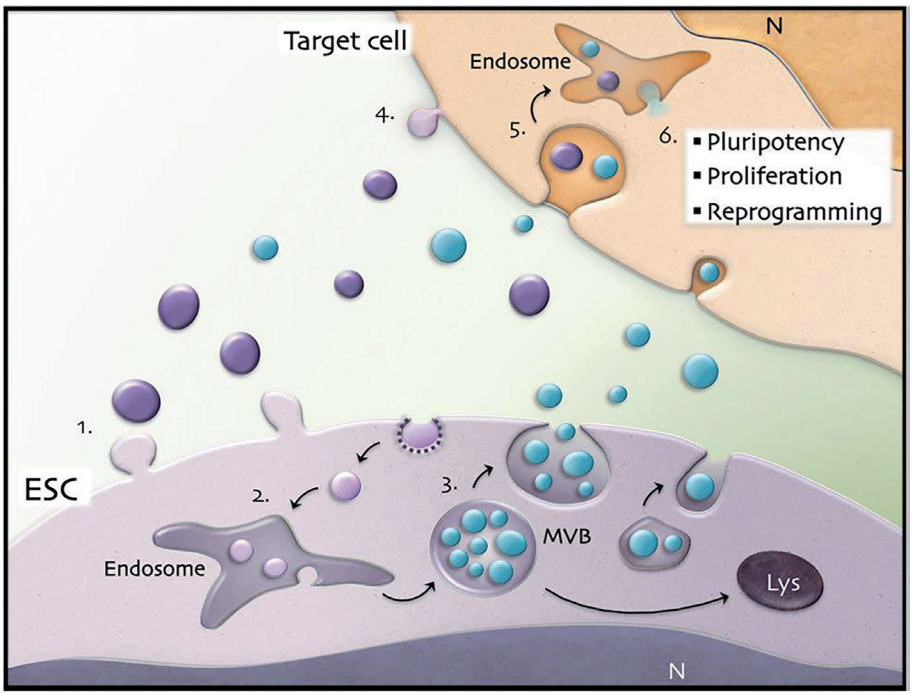

Figure 5. Extracellular vesicles as potential modulators of the stem cell base. Extracellular vesicles include microvesicles (magenta) released from the cell membrane (1), as well as exosomes (blue) located inside multivesicular bodies (MVBs) formed by endosomes (2). MVBs fuse with the membrane and release exosomes (3) or can be targeted for degradation via lysosomes. In the microenvironment, extracellular vesicles can reach the target cell and deliver their contents through plasma membrane fusion (4), or they can be internalized and fuse with the surrounding membrane of the endocytic compartment (5). The release of their contents inside the recipient cell can modulate the effects according to the state of the stem cells (6). N, nucleus; Lys, lysosome; ESC, embryonal stem cell (according to Cruz et al. (2018)).

and diameter) of particles released by IVF embryos differed from that released by parthenogenetic embryos. The data suggest that extracellular vesicles population characteristics differ depending on embryo competence.

Cytoskeleton

The cytoskeleton creates a structural support of trophectoderm and ICM cells. It serves for intracellular transport and movement of organelles and intracellular inclusions inside the cell. Filaments of actin cytoskeleton, demonstrated by electron microscopy in mouse oocytes, play an important role in fertilization (Karasiewicz and Soltyńska 1985). However, the cells of the first stages of embryo division up to the morula do not contain proteins of intermediate filaments (Traub 1985). Only higher stage of embryos, the blastocyst, has a well-developed cytoskeleton, consisting of microtubules, microfilaments, but mainly intermediate fibers (Maddox-Hyttel and Boerjan 2002), which are relatively well detectable by electron microscopic analysis.

Using a fluorescently labeled (TRITC) phalloidin, we found a regular distribution of actin filaments forming part of the cytoskeleton in the cytoplasm of trophectoderm and ICM cells in **morphologically normal blastocysts** (Fig. 6A).

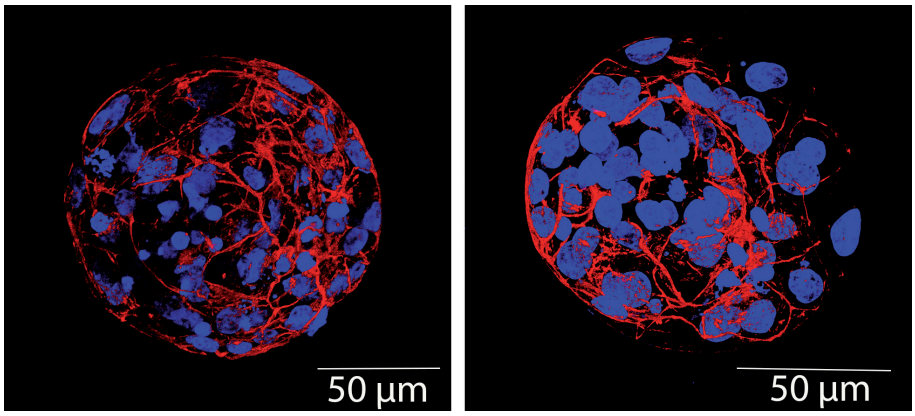

Figure 6. Fluorescent assay of bovine blastocysts. **A.** The actin cytoskeleton of the morphologically normal bovine blastocyst. The integrity of the trophectoderm layer is intact and the actin microfilaments in the cytoplasm of the cells form a regular network, ×600. **B.** The actin cytoskeleton of the morphologically abnormal bovine blastocyst is disintegrated in many places and forms numerous clusters. Disrupted integrity of the trophectoderm layer, fragments of the trophectoderm cells in the perivitelline space after degradation of tight junctions and actin microfilaments, ×600.

In **morphologically abnormal blastocysts**, a disrupted integrity and completeness of the trophectoderm layer can be observed. After the degradation of tight intercellular junctions and actin filaments, fragments of trophectoderm cells have been found in the PvS. The disturbed cytoskeleton was formed into clusters.

Structural systems of cells of morphologically abnormal early bovine embryos

The different levels of occurrence of structural cell systems in morphologically abnormal (grade 3) bovine embryos obtained *in vivo* are presented in Table 1. In comparison to morphologically normal (grade 1) embryos, an increased incidence of damaged mitochondria, lipids, multivesicular bodies and autophagolysosomes was found in abnormal embryos.

Blastocysts are surrounded by a more porous but still present *zona pellucida*. The PvS is expanded and contains numerous masses of detritus. Microvilli at the apical end of flat trophectoderm cells are short and gradually disappear (Fig. 7A). Pinocytotic vesicles are formed only sporadically. Short and few microvilli are related to a reduction in the absorptive capacity of blastocysts (Shamsuddin and Rodriguez-Martinez 1994). The PvS is expanded and filled with detritus and variously sized cell fragments (Fig. 7B). Junctional complexes on the lateral sides of the trophectoderm cells are well developed and still present. The disruption of junctions observed between ICM cells results in an expansion of the intercellular space and is similar to that seen in embryos produced *in vitro* (IVP) (Prather and First 1993; Crosier et al. 2001).

During the study of nucleus/nucleolus formation, differences in the allocation of nuclear proteins were observed in differently obtained bovine embryos (Laurincik et al. 2003). Both the i*n vivo* developed and IVP embryos showed the allocation of nuclear proteins to the fibrillary and granular compartments of the developing nucleoli during the 4^{th} cell cycle. However, parthenogenetic embryos showed delayed activation with the formation of fibrillary-granular nuclei during the 5^{th} cell cycle. By studying the remodeling of the nucleus after its transfer, Kanka et al. (1999) found that the primary vesicle appeared in embryos after nuclear transfer at the four-cell stage, which was appeared one cell cycle earlier than in control (intact) embryos. Only nuclear transfer-derived embryos reconstructed from activated cytoplasts underwent complete nucleolar remodeling. Nuclei contain few clumps of peripherally located heterochromatin, but it is mostly scattered throughout the whole nucleus (Fig. 7B). Nucleoli are also present; some of them change from compact to the scattered form. Similar chromatin structuring and reduction of newly synthesized RNA was reported in blastomere nuclei of abnormal bovine embryos (Pivko et al. 2002) or bovine early embryos after their gamma-irradiation with a dose of 2–4 Gy (Pivko et al. 2001, 2002). Free ribosomes, Golgi complexes and rough ER are only sporadically found in the cytoplasm of such embryos. The cytoplasm of trophectoderm cells contains mitochondria with disrupted cristae.

Compact morulae and IVP blastocysts have a reduced occurrence of mitochondria compared to *in vivo* embryos (Crosier et al. 2000, 2001). Damaged mitochondria have a declined number of cristae, which causes a reduced ability to metabolize lipids and produce energy, what may be related to an increase in lipid droplets of different sizes in the cytoplasm of embryos (Fig. 7C). Vesicles with different contents of lysosome-like bodies, such as multivesicular bodies and autophagolysosomes, are numerous in the cytoplasm of trophectoderm and ICM cells (Fig. 7C,D). Cytoplasmic vesicles (mentioned by the authors as "vacuoles", when in fact, they observed "vesicles") of bovine blastocysts often phagocytose autolyzed cellular matter (Linares and Plöen 1981). It has been recommended to consider cytoplasmic vesicles of trophoectoderm and ICM cells as abnormal (Enders et al. 1982) or as a phenomenon causing delayed developmental differentiation (Shamsuddin and Rodriguez-Martinez 1994).

When cultivating embryos *in vitro*, a microenvironment can be created that impairs their development. Bovine IVP blastocysts, compared to their *in vivo* embryos, were characterized by a lack of desmosomal junctions, a reduction in the population of microvilli, an increase in the average number of lipid droplets, electron-lucent mitochondria and an increased amount of detritus in the PvS and expanded intercellular space. These embryos also showed large intercellular cavities (Rizos et al. 2002). However, the quality of ovine blastocysts was significantly higher than that of bovine blastocysts produced under identical *in vitro* conditions, suggesting inherent species-specific differences in embryo quality between these two species.

Abe et al. (1999) reported that the addition of serum to culture media caused increased deposition of lipid droplets in blastomeres. This study showed large differences in ultrastructural features between morulae and blastocysts from serum-free and serum-supplemented cultures, suggesting that ultrastructural differences may reflect physiological characteristics of the embryos.

Some effects of microenvironment and micromanipulation on the ultrastructure of early embryos

Embryo derivation

When determining the relative volume of cell components using ultrastructural morphometric analysis of bovine em-

bryos after insemination by application of **intracytoplasmic sperm injection (ICSI)** or **parthenogenetic electrical activation (AC/DC),** we found that in the morphologically normal embryos produced *in vivo*, a greater part of the cell volume (82.93%) was occupied by the cytoplasm (Pivko et al. 2003). The relative volume of mitochondria, Golgi complex, lipids, vesicles and inclusion bodies was minimal. In parthenogenetic AC/DC-activated embryos, a relatively high percentage was represented by vesicles and lipids. ICSI-derived embryos, compared to *in vivo* or parthenogenetically activated embryos had the lowest volume of cytoplasm (58.33%), but higher volume of lipids and vesicles.

Ultrastructural studies showed that IVP blastocysts after fertilization with **sperm separated by flow cytometry** had variations in the number and structure of organelles such as mitochondria, rough ER and nuclear envelope (Palma et al. 2008). These morphological changes may be responsible for the impaired development observed in embryos fertilized by sex-sorted sperm.

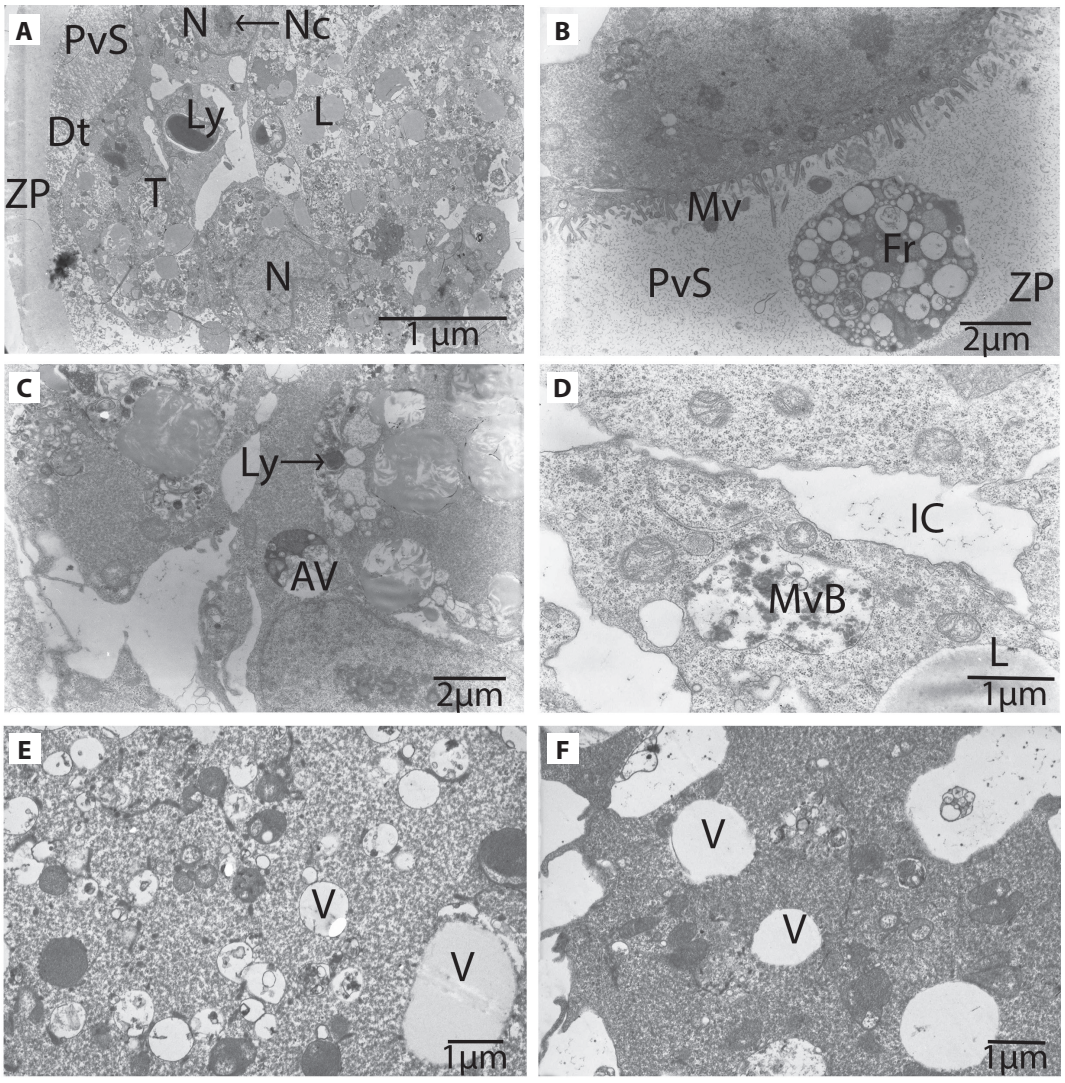

Figure 7. Ultrastructure of the morphologically abnormal bovine blastocyst (grade 3). **A.** *Zona pellucida* (ZP), expanded perivitelline space (PvS) filled with cellular detritus (Dt). Trophectoderm cells (T) with few microvilli. In the cytoplasm of trophectoderm and ICM cells, the appearance of lysosome-like bodies (Ly) and lipid inclusions (L). Nuclei with heterochromatin (N) and indistinct nucleoli (Nc), ×2000. **B.** A large cell fragment (Fr) in the perivitelline space (PvS) below the *zona pellucida* (ZP); microvilli (Mv), ×5000. **C.** A large autophagolysosomes (AV) and lysosome-like bodies (Ly) in the cytoplasm of the ICM cell, ×5000. **D.** A large multivesicular body (MVB) and a lipid droplet (L) in the ICM cytoplasm. Dilated intercellular spaces (IC), ×10 000. **E, F.** Vesicles (V) of various sizes in the cytoplasm, ×7200.

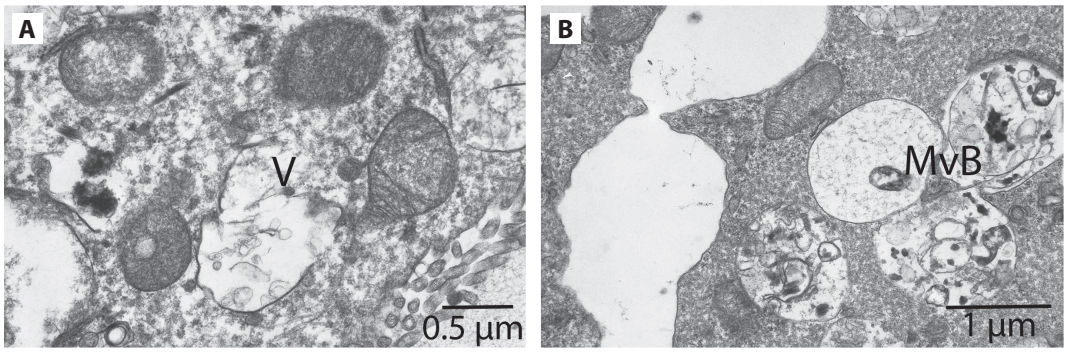

Figure 8. Ultrastructure of viral contamination of embryos. **A.** Vesicle formation in the trophectoderm cells (V) after infection of a blastocyst with bovine herpesvirus-1 (BHV-1). Photo from Makarevich et al. (2007). **B.** Increased occurrence of vesicles and multivesicular bodies (MVB) after infection of a blastocyst with the cytopathic strain (VDV-OREG, PhFTL 2) of BVDV virus, ×7200. Photo from Kubovičová et al. (2008).

Embryo cryopreservation

Bovine IVP blastocysts are extremely sensitive to cryopreservation. Thus, frozen IVP blastocysts showed disrupted *zona pellucida*, disintegrated cytoplasm, wider PvS, increased number of lipid droplets and loss of junctional contacts between trophoblastic cells (Fair et al. 2001). This study suggests that *in vivo* blastocysts have certain structural characteristics that give them greater tolerance to freezing. Embryos developed *in vivo* showed a rather modified ultrastructure after thawing with signs of osmotic changes in both trophectoderm and ICM. Darvelid et al. (1994) showed that *in vitro* and *in vivo* developed bovine embryos can survive vitrification using ethylene glycol as a cryoprotectant.

Viral contamination of embryos

Embryos infected with the **bovine herpesvirus-1 (BHV-1)**, which causes infectious rhinotracheitis in cows, showed significant vesicle formation in the trophectoderm cells, accumulation of viral particles in the cytoplasm around the nuclei and in the nucleoplasm (Bowen et al. 1985). Based on the direct immunofluorescence method using the polyspecific anti-IBR/IPV FITC conjugate, we found that the BHV-1 virus adheres to the *zona pellucida*, penetrates it and is localized mainly in trophectoderm cells, where it causes significant vesicle formation (Fig. 8A) (Pivko et al. 2003; Makarevich et al. 2007).

The ultrastructural morphology of the organelles of bovine embryos was also disturbed after sub-zonal microinjection of **bovine viral diarrhea virus (BVDV)** (Kubovicova et al. 2008), which causes mucosal disease and viral diarrhea in cows. After incubation with cytopathic BVDV strain (VDV-OREG, PhFTL 2), the embryos showed a positive signal for BVDV-fluorescent conjugate inside the embryo. Electron microscopy confirmed the increased occurrence of vesicles and multivesicular bodies (Fig. 8B).

Body condition of cows-embryo donors

Body condition of cows, evaluated by visual estimation of the body condition score (BCS), is an important factor, which can affect their reproduction ability (Bezdíček et al. 2020) through the changes in some essential cell organelles, revealed at the ultrastructural level. Thus, Olexikova et al. (2017) observed several differences in the ultrastructural morphology of preimplatation embryos recovered from over-conditioned cows (BCS4 and BCS5) in comparison to normally conditioned cows (BCS2 and BCS3). These differences were represented by different morphological patterns of mitochondria, occurrence of mitochondria with vesicles, the dilated intercellular spaces and higher lipid content in the cytoplasm (Olexikova et al. 2017). Changes in the appearance and localization of lipid droplets can reflect normal metabolism as well as certain pathological processes in the embryo (Awasthi et al. 2010; Bradley et al. 2016). Lipid droplets are in association with organelles linked to lipid metabolism, such as mitochondria, ER, endosomes and peroxisomes (Murphy et al. 2009). Therefore, higher lipid content in the cytoplasm of embryos can be a marker of their low quality and subfertility in over-conditioned cows (Olexikova et al. 2017).

Conclusion

Currently, a great deal of knowledge, according to the statement of Van Soom et al. (2003), can be deduced from the morphological observation of embryos, although not all. The reduced quality of bovine embryos produced under serum-supplementing conditions provided sufficient evidence to

link abnormal embryo morphology with more objective parameters of embryo quality. The main obstacle to the correct morphological assessment of the embryo viability currently lies in the impossibility of assessing the genetic makeup of the embryo in a non-invasive way. Progress in this field depends on adapting genetic analysis to the very small amount of a sample present in embryo biopsies and on correlating the genetic makeup of a biopsied material with the rest of the embryo.

Based on previous publications and our own results from electron microscopic and fluorescence analyses, we can state that the cells of the morphologically normal bovine blastocysts, classified as grade 1, have intact cell systems and are functional in a relatively optimal mode. Through the intensive process of pinocytosis, numerous vesicles containing nutrients for the developing viable embryo reach the cytoplasm, thus, ensuring the synthesis of the signal product, interferon-τ – a specific protein that stimulates the *corpus luteum* to protect pregnancy. In this context, extracellular vesicles also play an important role. In the cells of morphologically abnormal bovine embryos, classified as grade 3, these systems have varying degrees of impaired morphological integrity and functionality. In these cells, the onset of morphological deviations from the norm and degenerative processes can be observed. The number of lysosome-like bodies, multivesicular bodies, autophagolysosomes and lipid droplets increases and accumulates inside the cells, what leads to the formation of cellular debris, larger fragments limiting further development up to the death of the embryos. Most preimplantation embryos are mosaic, containing both normal and abnormal cells, and apoptosis may represent a checkpoint to eliminate such cells. In practice, this early embryonic mortality significantly reduces the fertility of cows, but currently we cannot determine, whether it is caused by abnormalities during the development of the embryos or by abnormal conditions of the uterine environment. Based on analyzes of the ultrastructure of the trophectoderm and ICM cells and all six functional systems, pre-implantation embryos can be more accurately classified into quality grades and, thus, their viability can be more accurately characterized in relation to fertility disorders as well as during embryo transfer.

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