NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Slovak J. Anim. Sci., 54, 2021 (4): 162–167
ISSN 1337-9984, E-ISSN 1338-0095

ULTRASTRUCTURAL MORPHOLOGY OF CELL ORGANELLES IN BOVINE VITRIFIED OOCYTES

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ABSTRACT

Oocytes during cryopreservation are exposed to adverse conditions and factors that result in various damages reducing their developmental capacity. Most of such lesions may not be visible to light microscopy when assessing morphology. The aim of our study was to examine the ultrastructure of bovine *in vitro* matured (IVM) oocytes following cryopreservation. IVM oocytes were vitrified using ultra-rapid cooling technique in minimum volume on the electron microscopy grids and stored in liquid nitrogen for several weeks. After warming the oocytes were fixed, dehydrated and embedded into resin. Ultrastructure of oocytes was analysed on ultrathin sections obtained from embedded oocytes. Several alterations and damages to cell organelles of vitrified oocytes (smooth endoplasmic reticulum (SER), mitochondria and lipid droplets) were revealed in contrast to the fresh oocytes. Some membranes of large vesicles of SER were damaged and vesicles were obviously fused. Similarly, fusion of small lipid droplets to form large lipid droplets was visible in vitrified/warmed oocytes. Mitochondria showed signs of slight vacuolation, loss of mitochondrial matrix or less recognizable cristae. Differences were found also in the cortical area of oocytes (cortical granules, oolemma, *zona pellucida* and microvilli). However, these damages were less extensive than were presented in vitrified bovine oocytes previously, what indicates the suitability of our vitrification technique. In conclusion, ultrastructure assay can reveal individual membrane damages to organelles inside the oocyte, what may help in explaining developmental failures of oocytes following vitrification and warming.

Key words: bovine oocyte; IVM; ultrastructure; vitrification

INTRODUCTION

Vitrification is an important method for application in cattle breeding and conservation of animal genetic resources. The use of a suitable combination of cryoprotectants together with a very high cooling rate will ensure the survival and viability of the most cryopreserved oocytes. However, during freezing, oocytes are always exposed to adverse conditions, which result in various damages to the oocytes (Hyttel *et al.*, 2000; Diez *et al.*, 2005; Morató *et al.*, 2008).

The state and quality of oocytes after warming is usually assessed by a light microscopy using morphological criteria or after fluorescent staining of individual compartments. The basic method is an evaluation of embryo development after *in vitro* fertilization of vitrified oocytes. Nowadays, also other methods revealing metabolic, genomic and other features of oocytes or early embryos have a growing popularity (Goovaerts *et al.*, 2010). However, electron microscopy, by observing at very high magnification, can detect details of fine structures inside cells and damage at the level of

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Received: November 4, 2021
Accepted: November 22, 2021

individual membranes. Although this method is cost- and time-demanding for sample preparation and, therefore, is not widely used over the world, this assay could offer a unique and valuable information about the intrinsic state of cells, which is hidden for commonly used evaluation techniques.

The aim of our work was to study the ultrastructural state of cryopreserved *in vitro* matured bovine oocytes after vitrification and warming.

MATERIAL AND METHODS

All the chemicals used in this study were purchased from Sigma-Aldrich Inc. (Saint-Louis, Missouri, USA), unless otherwise indicated.

Oocyte retrieval and *in vitro* maturation (IVM)

The ovaries were isolated from undefined cows at a local abattoir and transported to the laboratory at ambient temperature. The oocytes were recovered from antral follicles (2 – 8 mm) by the aspiration of follicular fluid using sterile 5 mL syringe with a needle (18 gauge). Cumulus-oocyte complexes (COCs) were collected into a Petri dish with a holding medium (M199-HEPES with 10 % foetal bovine serum – FBS) and only COCs with several layers of cumulus cells and homogeneous ooplasm were selected for IVM. COCs intended for vitrification were matured for 21 h, while those selected for the control group were matured for 23 h in a maturation medium containing M199 (Gibco), sodium pyruvate (0.25 mmol.L^{-1}), gentamycin (50 mg.mL^{-1}), 10 % FBS and FSH/LH (1/ 1 I.U., Pluset) at 38.5 °C and 5 % CO_2 in the incubator.

Cryopreservation of oocytes

For cryopreservation of IVM oocytes, ultra-rapid cooling technique in minimum volume was used as described previously (Olexikova *et al.*, 2019). Briefly, selected IVM oocytes were stripped off an excessive cumulus layers by vortexing for 30 s. Oocytes with approx. three remaining cumulus cell layers were placed into equilibration solution (ES; 3 % ethylene glycol (EG) in M199-HEPES) supplemented with 10 % FBS, for 12 min. Following equilibration, the oocytes were transferred to vitrification solution (VS; 30 % EG; 1 M sucrose in M199-HEPES with 10 % FBS) at room temperature for 25 s. The oocytes (10 – 15) in a small drop were transferred onto 300 mesh nickel electron microscopy (EM) grids with a glass micropipette. An excessive medium was removed by a filtration paper and then the oocytes were immediately plunged into liquid nitrogen for storage (several weeks).

For warming, nickel EM grids were transferred directly into warming solution (0.5 M sucrose in M199-HEPES, at 37 °C) for 1 min. The warmed oocytes were passed across the three diluent solutions with decreasing sucrose concentrations (0.25 M, 0.125 M and 0.0625 M sucrose in M199-HEPES) for 3 min in each, and then washed twice in M199-HEPES with 10 % FCS for 5 min. Oocyte survival was evaluated using light microscopy inspection on the basis of the integrity of the ooplasm and the *zona pellucida* after 2 h culture post-warming. Oocytes with intact oolemma, intact *zona pellucida* and homogenous and dark cytoplasm were considered as surviving oocytes.

Ultrastructure assay of oocytes by transmission electron microscopy

Oocytes from vitrified and control groups (10 per group) were randomly selected for ulstrastructure assay. Control oocytes were selected after IVM and vitrified oocytes were selected after warming. Selected oocytes were immediately fixed in an aldehyde mixture (2.0 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3) at 4 °C for 60 min, then washed twice in 0.1 M sodium cacodylate buffer for 5 min. Subsequently, the oocytes were individually embedded into 2 % agar and post-fixed in 1 % OsO_4 in 0.1 M sodium cacodylate buffer for 1 hour at room temperature. The samples were then dehydrated by passing them through an acetone series, embedded into Poly/Bed resin (Polysciences Inc., Warrington, USA) and serially sectioned on Ultracut UC 6 ultramicrotome (Leica) into semi-thin sections (1 µm), which were stained with toluidine blue for light microscopy. The ultra-thin sections (70 nm) were collected on nickel grids, contrasted with uranyl acetate and lead citrate, and examined on a JEOL CX100 transmission electron microscope (JEOL, Tokyo, Japan). The ultrastructural characteristics of the cells were compared between the vitrified group and the control.

RESULTS AND DISCUSSION

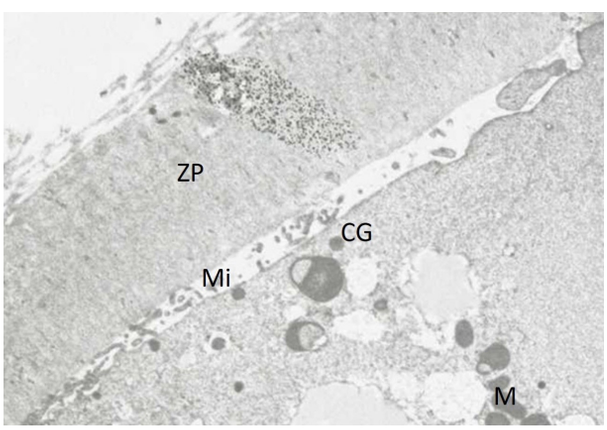
Ultrastructure of vitrified /warmed bovine oocytes has been studied earlier (Fuku *et al.*, 1995; Hyttel *et al.*, 2000; Diez *et al.*, 2005; Morató *et al.*, 2008; Sprícigo et al, 2014; Olexiková *et al.* 2020). Authors revealed several damages and alterations in contrast to the fresh oocytes. Major differences were found in cortical region of oocytes, but also other organelles, such as smooth endoplasmic reticulum, mitochondria, lipid droplets; partially cortical granules were affected by vitrification. The main compartments of the oocytes, where damages more often occur during vitrification/warming are described in detail below.

Zona pellucida and cortical granules

Zona pellucida is a mucopolysaccharide layer surrounding the oocyte and preimplantation embryo up to the hatching stage. It participates in fertilization processes, plays a role in the interaction of oocyte with sperm and forms a protective barrier with the irreplaceable role of a block against polyspermia. Fuku *et al.* (1995) demonstrated changes in the *zona pellucida* quality defined as zona hardening as a consequence of cortical granule release. In our vitrified oocytes no signs of cortical granule release were noted, and the *zona pellucida* of oocytes from both the experimental and control groups appeared to have the same structure (Figure 1). Similar to our findings, some authors also did not observe any changes in cortical granule release in mouse and human oocytes (Gook *et al.*, 1993; Van Blerkom and Davis, 1994). However, Hyttel *et al.* (2000) documented alterations in cortical granule distribution and their degradation in some cases after vitrification.

Oolemma

Surface membrane surrounding the whole oocyte – oolemma, forms microvilli, which increase the resorption area of the oocyte. It is a place of contact with the environment as well as a place of interaction with sperm. It plays a role in the uptake and excretion processes by endocytosis and exocytosis. Status of the cell surface, quality and disruption of the cytoplasmic membrane or microvilli disruption is one of the basic indicators of oocyte quality and damage after warming. Disturbance of the oolemma integrity is often associated with a disruption, partial lack or complete absence of the microvilli. Notola *et al.* (2009) in their study with human oocytes found that in 30 % of vitrified-warmed oocytes, surface areas with rare and short microvilli were found. Fuku *et al.* (1995) also showed that vitrified oocytes exhibited changes in the microvilli and formation of vesicles. Accumulation of such vesicles on the periphery of ooplasm resulted in the rupture of plasma membrane in many oocytes. However, in normal mature oocytes such vesicles were not present. Hyttel *et al.* (2000) also reported the appearance of small membrane-bound vesicles in oocytes examined immediately after warming. In some cases, a rim of vesicles had become located along the oolemma. Subsequently, in vitrified oocytes and zygotes examined at 4 hr after warming, the small membrane-bound vesicles had become fewer, probably as a sign of regeneration. Finally, in oocytes and zygotes examined at 26 hr after warming, small vesicles were almost absent. In contrast to Fuku *et al.* (1995), we observed a clearly visible zone without organelles on the periphery of the warmed oocytes, where the occurrence of vesicles was only occasional. The cytoplasmic membrane appeared intact with a normal microvilli presence (Figure 1).



Cortical area of bovine IVM oocyte after vitrification/warming. Visible *zona pellucida* (ZP), microvilli (Mi) in the perivitelline space. Cortical granules (CG) under the oolemma in solitary formation. Primary magnification of figure is x2000.

Figure 1. Vitrified/warmed bovine oocyte

Smooth endoplasmic reticulum (SER)

In the cell, SER is the site where the synthesized products are transformed, completed, transported and stored. It is closely related to the metabolism and post-translational modifications of proteins, but also to the metabolism of lipids in the oocyte. Functionally, therefore, it is closely related to mitochondria and lipid droplets. It is formed by connected cisterns and vesicles forming a network of membranes throughout the cytoplasm of the oocyte. In our vitrified oocytes some membranes of large SER vesicles were damaged and vesicles appeared to be fused (Figure 2). Notola *et al.* (2009) in their study with human oocytes found that in about 50 % of the vitrified-warmed oocytes, the mitochondria-SER aggregates appeared changed in size and shape compared to those observed in fresh oocytes.

Lipid droplets (LDs)

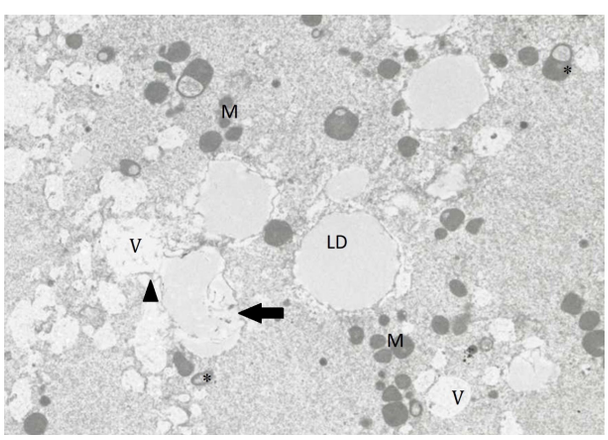
As already mentioned, LDs are closely related to the function of SER and mitochondria. LDs are not only balls for the lipid storage. Several proteomic analyses of LDs revealed high variability of their protein composition that probably reflects different functional roles of LDs (Hodges *et al.*, 2010), and LDs represent dynamic structures involved in specific functions in cells (Hapala *et al.*,2011; Ohsaki *et al.*, 2014). Thus, damages in LD structures may affect an oocyte and may cause multiple function impairments. Similar to SER vesicles, there was a visible fusion of small LDs to form large LDs in our vitrified/warmed oocytes (Figure 2).

Mitochondria

Mitochondria as the energy generator of the cell are among the most sensitive organelles, highly susceptible to damage. As a site of active oxidation, it is behind the production of radicals in the cell, and the release of their components while disrupting their membrane system has the potential to damage the whole cell. In the studies of Fuku *et al.* (1995), Hyttel *et al.* (2000), Diez *et al.* (2005), Morató *et al.* (2008) and Sprícigo *et al.* (2014), certain changes or damages to the oocyte mitochondria were observed after vitrification and warming. These changes in our warmed oocytes included slight vacuolation of mitochondria, loss of mitochondrial matrix and less recognizable cristae (Figure 2). Mitochondria are still located closely adjacent to the SER vesicles, and their distribution and location is considered to be normal.

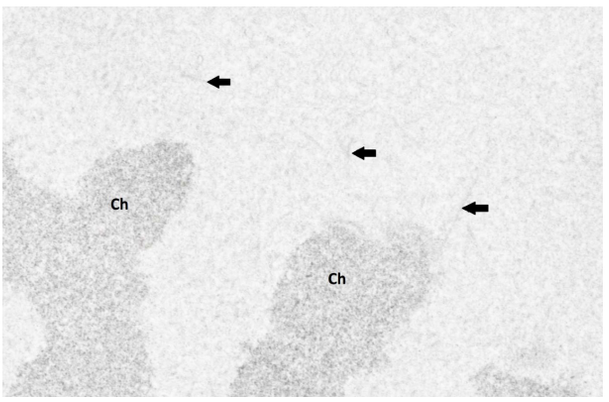
Chromatin

Sprícigo *et al.* (2014) reported, that only 55 % of the oocytes vitrified at the end of IVM, were stayed at the MII stage after warming, most likely due to intense chromatin degeneration in response to vitrification. These results suggested that the oocytes were severely affected by vitrification procedure and exhibited high degree of chromatin degeneration and abnormality. In contrast to these findings, all our vitrified/warmed IVM oocytes were at the MII stage. However, disturbing of the metaphase plate arises not only from chromatin damage but mainly from meiotic spindle damage. Exactly, cytoskeletal damage and tubulin depolymerization is one of the most commonly described consequences of oocyte cryopreservation at the metaphase stage. It was documented that the critical damage after oocyte vitrification is spindle disorganization caused by the disassembly of microtubules (Albarracin *et al.*, 2005). We also observed the partial loss of microtubules around the chromosomes in some vitrified oocytes. However, in most warmed oocytes microtubules were still present (Figure 3).



Ooplasm of bovine IVM oocyte after vitrification/warming. Visible mitochondria (M) in some cases show signs of matter loss and slight vacuolization (asterisk). The vesicles (V) of the smooth endoplasmic reticulum have damaged membranes and are occasionally fused (arrowhead), similarly as lipid droplets (LD; arrow), in the ooplasm of vitrified oocytes. Primary magnification of figure is x2000.

Figure 2. Ooplasm of vitrified/ warmed bovine oocyte



In the vitrified oocyte, numerous microtubules of the meiotic spindle (arrows) are still visible around the chromosomes (Ch). Primary magnification of figure is x10000.

Figure 3. Microtubules in vitrified/ warmed bovine oocyte

Based on the described alterations in the ultrastructure, it is evident how the oocytes were affected by vitrification. However, the extent of injury in our vitrified oocytes is lower than that revealed by other authors studying the ultrastructure of cryopreserved oocytes. This is also supported by the improved development of our oocytes after IVF, where approximately 17 % of them developed to the blastocyst stage (Olexiková *et al.*, 2020), as opposed to the aforementioned reports, where the blastocyst rates were ranged from 0.0 to 4.6 % (Fuku *et al.*, 1995; Diez *et al.*, 2005; Sprícigo *et al.*, 2014).

CONCLUSION

The ultrastructural pattern of vitrified/ warmed oocytes in different studies showed to be very different. Serious damages at the ultrastructure level revealed by some authors were also associated with a greatly reduced developmental capacity of these oocytes. The extent of the damage as well as the subsequent developmental capacity probably depends on the cryopreservation methodology and the suitable vitrification procedure. Ultrastructure assay can reveal individual membrane damages to organelles inside the oocyte, what may help in explaining developmental failures of oocytes following vitrification and warming.

ACKNOWLEDGMENTS

This study was supported by the Slovak Research and Development Agency of the Slovak Republic (grant no. APVV-19-0111).

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