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Viability of bovine in vitro matured oocytes following ultra-rapid vitrification

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Abstract

The aim of the study was to examine viability of cattle oocytes after cryopreservation. Oocytes after *in vitro* maturation (IVM) were vitrified in minimum volume on the nickel electron microscopy grids by ultra-rapid cooling technique. After warming and subsequent in vitro fertilization the presumptive zygotes were cultured to reach the stage of the blastocyst (Bl). Several devitrified oocytes were processed for electron microscopy assay. Although, embryo cleavage and Bl percentages in the vitrified group were slightly lower than in the control group ($P < 0.05$), the Bl total cell number (TCN), apoptosis and dead cell percentages did not differ between both groups. However, significant difference was found between day 7 (D7) and day 8 (D8) Bl in the TCN in control (108.0 vs. 90.5) and vitrified group (103.75 vs 98.14). Electron microscopy of frozen oocytes revealed slight reversible injuries in mitochondria and the smooth endoplasmic reticulum (SER), nevertheless, the development of devitrified oocytes to the Bl stage was comparable to those in fresh oocytes. In conclusion, higher proportion of slower developing Bl (D8) compared to D7 Bl may be related to the mentioned minor damages of some organelles in vitrified oocytes.

Running title: Viability of bovine vitrified oocytes

Keywords: vitrification, bovine, oocyte, embryo, ultrastructure, apoptosis

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Introduction

Long-term preservation of genetic material represents crucial tool in reproduction nowadays. New methods and modifications are constantly being developed for better survival of gametes and embryo development after cryopreservation. Unfortunately, there is still no reliable procedures to eliminate injuries during cryopreservation ensuring higher embryo development and morphologically normal blastocysts.

Vitrification reduces the formation of intracellular crystals, restores oocyte volume more rapidly, increases survival and preserves spindle structure compared to slow freezing [1]. However, vitrification requires much higher concentrations of cryoprotectants than slow freezing, which can be toxic to cells if are improperly treated or exposed for too long. Nevertheless, despite these difficulties, vitrification increases survival after warming and the number of morphologically normal cells [2]. Cryopreservation of bovine oocytes has still low developmental rate after warming, mainly due to the high sensitivity to the formation of intracellular ice crystals, the content of lipid droplets in the cytoplasm [3] and the lower surface-to-volume ratio [4]. Abnormal distribution of mitochondria, cytoplasmic degeneration [5], cytoskeleton or microtubule degeneration [6] occurs during vitrification, resulting in an increased occurrence of apoptotic and dead cells in developing embryos. This is subsequently reflected in lower quality of *in vitro* produced (IVP) embryos.

This study was aimed to assess the viability of cattle oocytes after cryopreservation determined as ultrastructural morphology of oocyte organelles, as well as blastocyst rate, cell number and apoptotic/dead cell ratio following *in vitro* fertilization (IVF) and culture.

Materials and Methods

Oocyte collection and in vitro maturation

The ovaries for the experiments were obtained from slaughtered cows from the local abattoir and processed according to Olexikova et al. [7]. Briefly, oocytes were aspirated from the follicles with a size of 2-8 mm. Only suitable cumulus-oocyte complexes (COCs) with a homogenous cytoplasm and the intact cumulative cell layer were chosen for IVM (TCM199 (Gibco), 0.25 $mmol.l^{-1}$ of sodium pyruvate, 50μg/ml gentamicin, 10% foetal bovine serum (FBS) and 1/1 I.U FSH / LH (Pluset)) at 5% CO_2 and 38.5°C for 21 hours in the case of an vitrified group consisting of embryos obtained from vitrified oocytes. The control group of fresh oocytes was matured *in vitro* for 23 hours.

Oocyte vitrification and warming

The experimental vitrified group of oocytes after *in vitro* maturation was incubated in the equilibration medium (M199, 10% FBS, 3% ethylene glycol-EG) for 12 min. Equilibrated oocytes were subsequently washed in the vitrification medium (M199, 10% FBS, 30% EG, 1M sucrose) for 25 sec. Groups of 10-15 oocytes were placed onto electron microscopy grids (EMGs); an excessive medium was aspirated using filter paper and the EMGs were immediately placed into a small box with liquid nitrogen. Such vitrified oocytes were then placed into a cryotube (2 ml) for better handling, and stored in a liquid nitrogen container for at least one week. Afterwards, oocytes were warmed by inserting EMGs into warming medium (M199, 0.5M sucrose, 10% FBS, 50μg/ml gentamicin) for 1 min at 37°C. Warmed oocytes were washed in a set of media with decreasing sucrose concentrations (0.25; 0.125; 0.625 M) for 3 min and then in a medium without cryoprotectant (M199-HEPES + 10% FBS) and incubated in the maturation medium at 38.5°C and 5% CO_2 for 2 hours.

In vitro fertilization and embryo development

Oocytes selected for IVF were washed, according to Olexikova et al. [7], in IVF-TALP medium (Tyrode-Albumin-Lactate-Pyruvate solution containing 10μg/ml heparin and 50μg/ml gentamicin) and placed into previously prepared fertilization drops covered with 850μl mineral oil, to which 3μl of PHE (20μM penicillamine, 10μM hypotaurine and 1μM epinephrine) solution and sperm suspension (2×10^6/ml) were added. Following 18-hour incubation presumed zygotes were cleaned of excessive cumulus cells and placed into an *in vitro* culture dish consisting of B2 Menezo medium (with 10% FBS, 10mg/ml BSA, 31.25mM sodium bicarbonate and 50μg/ml gentamicin) in co-culture with BRL (Buffalo Rat Liver) cells at 38.5°C and 5% CO_2. *In vitro* development of embryos was monitored on the day 2 (cleavage), day 7 and day 8 (blastocyst rate).

Ultrastructural assay

Randomly selected oocytes from each tested groups (n=10 per group) were prepared for an ultrastructure assay. Oocyte preparations were serially sectioned into semi-thin sections (1 μm) on an Ultracut UC٦ ultramicrotome (Leica) and stained with toluidine blue for light microscopy. Ultra-thin sections were analysed on a transmission electron microscope (JEOL CX100, JEOL, Tokyo, Japan). The ultrastructural images of oocytes were acquired and their morphology was compared among tested groups.

Fluorescent staining

Selected D7 and D8 blastocysts were fixed in 4% neutrally buffered formalin for 10 min and washed 3 times in PBS-PVP (phosphate-buffered saline with polyvinylpyrrolidone).

Dead cells with a damaged membrane were revealed by staining the embryos with a red fluorescent

dye - propidium iodide (PI; 25μg/ml; BD Pharmingen™) using a 5 min incubation (37°C at 5% CO_2) and subsequent three times washing in PBS-PVP.

The number of apoptotic cells was assessed by counting TUNEL-positive (Dead End Fluorometric TUNEL system; Promega, USA) cells. Fixed blastocyst (4% formalin) were washed in PBS-PVP, permeabilized in 0.5% Triton X-100 for 60 min at room temperature and then incubated in a TUNEL reagent mixture (according to the manufacturer) in humidified chamber at 37°C for 60 min and thereafter washed in PBS-PVP.

Stained blastocysts were placed onto coverslips, covered with a 2 μl of Vectashield anti-fade medium containing DAPI fluorochrome (Vector Laboratories, Burlingame, CA, USA) for labelling the blastocyst nuclei, attached to microslides to make a sandwich and analysed using a confocal microscope (Zeiss LSM 700).

Statistical analysis

The results from each experiment consisted of 6 replicates. The obtained data were statistically evaluated by a SigmaPlot 11.0 software. The cleavage and the blastocyst rates separately for D7 and D8 embryos were evaluated using the Chi-square test. Rates of apoptotic and dead cells were obtained as percentages from the total cell number. Differences in the cell numbers, apoptotic and dead cells between day 7 and day 8 blastocysts among the tested groups were evaluated by Student´s t-test. The level of significance of the tests was determined at $P < 0.05$.

Results

In our study, from 500 vitrified-warmed oocytes, 75% of oocytes survived warming procedure in good quality. About 400 fresh oocytes used for IVF were assigned as a control group. Cleavage rate was evaluated on the day 2 (D2) and blastocyst rates were evaluated on day 7 (D7) or day 8 (D8) following IVF. Significant differences between the control and vitrified group were found in cleavage (71% vs 65%) and blastocyst rates (26% vs 17 %). Differences in the rate of blastocyst formation on day 7 and 8 were found between the groups. In particular, 72.5% of embryos in the control group reached the blastocyst stage on the day 7 versus 57% in the vitrified group (**Fig. 1**). On the other hand, percentage of embryos reaching the blastocyst stage on the day 8 was significantly higher in vitrified group (43%) than in control (27.5%).

The results of the quality parameters of the D7 and D8 blastocysts are shown in **table 1**. No significant differences between the control and vitrified group in D7 blastocysts in the total cell number (TCN), apoptotic (TUNEL) and dead cells (PI) were found. Similar results were obtained for D8 blastocysts. However, the embryos that have reached the blastocyst stage earlier (D7), contained significantly more cells than those reached the blastocyst stage later (D8) in both control and vitrified groups. Bovine oocytes after warming showed some ultrastructural differences in several organelles in comparison with fresh oocytes (**Fig. 2A, 2B**). The ooplasm was denser and darker. No signs of cortical

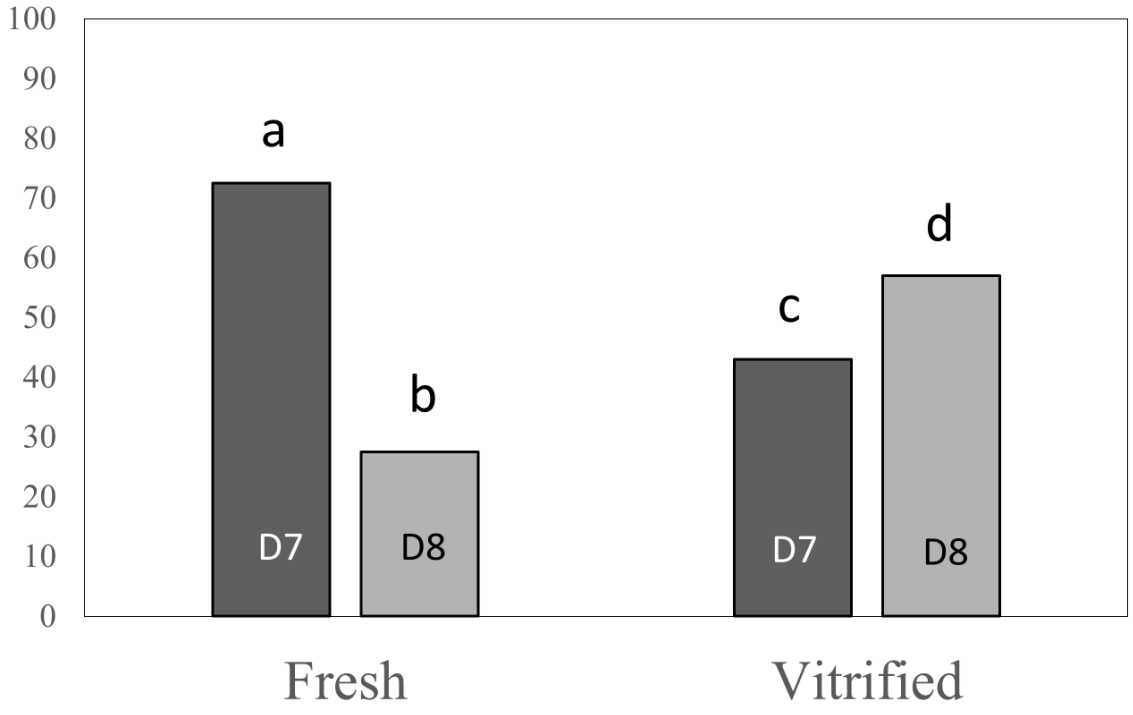


FIGURE 1 The blastocyst rate on day 7 and day 8 from fresh and vitrified oocytes. a,b,c,d – values with different letters above bars differ significantly: a vs. b – $P < 0.001$; c vs. d – $P < 0.05$; a vs. c – $P < 0.001$; b vs. d – $P < 0.001$ (Chi-square test)

TABLE 1 Effect of oocyte vitrification on quality of day 7 and 8 blastocysts (mean ± S.E.M.)

GROUP	EVALUATED BLASTOCYSTS, N	TOTAL CELL NUMBER	TUNEL INDEX (%)	PI INDEX (%)
Fresh D7	26	108.00 ± 5.03[a]	7.23 ± 0.83[a]	3.92 ± 0.75[a]
Fresh D8	24	90.50 ± 5.24[b]	8.44 ± 0.86[a]	4.00 ± 0.67[a]
Vitrified D7	12	103.75 ± 7.41[a]	7.30 ± 1.22[a]	5.19 ± 0.94[a]
Vitrified D8	14	95.08 ± 7.12[b]	9.87 ± 1.40[a]	5.74 ± 1.05[a]

a, b – values with different superscripts in the same column are significantly different (P<0.05)

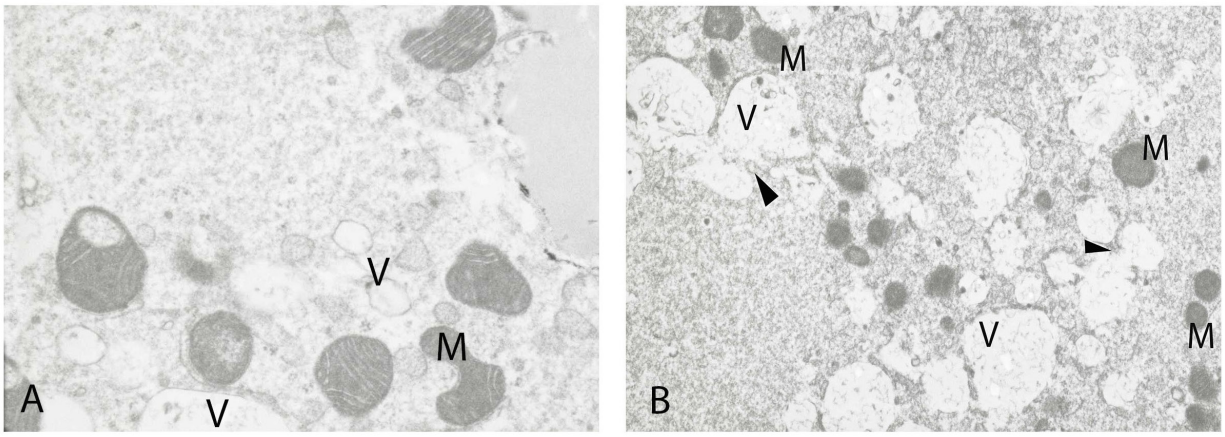


FIGURE 2 Ultrastructure of the ooplasm in fresh (control group, A) and vitrified/warmed (B) bovine in vitro matured oocyte. In the cytoplasm, there are abundant mitochondria (M) and vesicles (V) of the endoplasmic reticulum. In vitrified oocytes (Fig. 2B) the mitochondria are denser. The damaged mitochondria are vacuolated and their cristae are less distinguishable. The vesicles of the smooth endoplasmic reticulum are linked (arrowheads) and contain damaged membranes. Primary magnification of the figure - x10000

granule release or hardening of the *zona pellucida* were noted on the oocyte periphery. In the ooplasm, smooth endoplasmic reticulum has been partially disturbed and membranes of vesicles are damaged. These damaged vesicles of smooth endoplasmic reticulum are occasionally fused together (**Fig. 2B**). Mitochondria in vitrified group showed several damages including loss of mitochondrial matrix and vacuolization. Cristae of mitochondria in vitrified oocytes were less prominent than in the control. All changes observed in the ultrastructure of the vitrified oocytes after warming were only minor and did not substantially compromise further embryo development.

Discussion

The embryo development after oocyte vitrification, as a leading technique used for long-term storage of genetic material, is still insufficient. Expanding knowledge of oocyte damage caused by process of vitrification is necessary for methodology optimization. Therefore, our goal was to compare the embryo development and quality in vitrified and fresh groups of embryos.

Our present study showed significant difference in blastocyst rate between vitrified and fresh group (17.29% vs. 25.5%). Gutnisky et al. obtained only 8.8% blastocysts from vitrified partially denuded bovine oocytes compared to the fresh oocytes (18.2%) [8]. Magnusson et al. obtained from 4.17 to 8.16% of blastocysts from IVM vitrified oocytes and from 15.31 to 18.09% of blastocysts from immature vitrified oocytes compared to 51.6% blastocysts in non-vitrified control [9]. The results of various studies are variable, mainly due to different laboratory conditions and varying initial quality of oocytes.

Electron microscopy grids (EMGs) as a vitrification carrier have also been used earlier [10-12]. Blastocyst yields from cryopreserved oocytes reported in these studies are also very variable ranging from 3.61 to 20%. However, Matsumoto et al., who also used EMGs, was not successful in obtaining blastocysts (0%) from vitrified oocytes [13].

Higher total cell number was observed in embryos that have reached blastocyst stage on day 7 (103.75–108.5) than on day 8 (D8; 90.5-95.08) in both fresh and vitrified groups. Similar cell counts (84.3-98.1) have been reported by other authors [14,15] in D7 blastocysts derived from vitrified oocytes. Knitlova et al. obtained a higher total cell count (129 ± 24.3) than our study, using the same culture medium (B2) and culture conditions, but

they evaluated blastocysts on the day 8, what could explain a difference in this parameter compared to our data [16]. Data on the embryo cell counts are often incomparable, as some authors report the total cell number of all obtained blastocysts, while others [17] divide the results according to the stage of blastocysts (normal, expanded or hatched). In our study, no significant differences were found in quality parameters of D7 and D8 blastocysts (total cell number, apoptotic and dead cell rates) between control and vitrified groups. This observation suggests that the quality of blastocysts obtained from vitrified oocytes was comparable to those from fresh oocytes. However, there were significant differences in the total cell number between D7 and D8 blastocysts in both control and vitrified groups (**Tab. 1**). Our results are in agreement with the report of Markkula et al. that vitrified bovine D7 blastocysts contained more proliferating (PCNA-positive) cells than D8 blastocysts [18].

The number of apoptotic and dead cells was only slightly higher in the D8 than in the D7 blastocysts, as the difference was not statistically significant. The apoptotic cell ratio in D7 blastocysts from both groups was about 7%, what is comparable to the study of Foulandi-Nashta et al. [19]. Neuber et al. in their study obtained lower percentage of apoptotic cells (4%) than in our study (7.23%) [20]. Paschoal et al. obtained a much higher percentage of apoptotic cells in blastocyst from fresh oocytes (12.1%) and, after their vitrification and warming, observed significant increase in apoptotic cell ratio (22.3%) [21]. The percentage of dead cells (PI-index) as well as the number of apoptotic cells did not differ significantly between the control and vitrified groups, although a slight increase in the number of dead cells in the vitrified group compared to the control fresh group is evident. The dead cell percentage in our study is (4%) is comparable to those of Huang et al. [22], but is more than twice lower (9.8%) than reported by Hellmond et al. [23].

The ultrastructural damage is one of the most adverse events associated with cryopreservation due to cryoprotectant toxicity, ice crystal formation and osmotic stress [24].

We observed several changes in vitrified oocytes, which, however, were not very extensive and, thus, did not cause extensive oocyte damage. Premature cortical granule release has also been described by other authors [25-27] as a result of cryoprotectant exposure. Cortical granule release into the perivitelline space may results in hardening of the *zona pellucida* [28]. We observed mild damage to the cortical granules but with no signs of their release or *zona pellucida* hardening in vitrified oocytes. Similar results were obtained by Gook et al. in their study on human oocytes [29]. Similarly, Van Blerkom and Davis reported cortical granule release in only a small portion (5%) of mouse oocytes after vitrification [25].

In our vitrified oocytes, tubules of SER formed aggregates in the cortical area with slightly damaged membranes of vesicles. Pavani et al. described, that profiles of SER in mature bovine oocytes were scattered in the ooplasm, where they formed larger aggregates in the otherwise organelle-free ooplasmic regions [30]. The presence of large SER aggregates in human oocytes caused a developmental impairment, because of reduced release of calcium, which plays a key role during fertilization and early embryo development [31]. This may be a cause of reduced embryo production from vitrified oocytes.

It was reported [32-35], that vitrification process causes changes in the mitochondrial morphology and functions in bovine oocytes. The ultrastructure often shows reduced matrix density in mitochondria with vacuolization and membrane damage [36,33] which were confirmed in our study. Nevertheless, organelle alterations in warmed oocytes revealed in our study seem to be reversible, and some post-warm incubation is needed to restore injuries. Therefore, slower development of vitrified/warmed oocytes compared with fresh control may be a consequence of such restoration period. Higher rate of the slower developing (D8) blastocysts is probably related to the mentioned alterations. Nevertheless, further development devitrified oocytes to the blastocyst stage was not greatly compromised.

Conclusions

Vitrified oocytes in our experimental design resulted in slightly lower blastocyst rate than fresh oocytes, with the prevalence of slower developing blastocysts, which may be related to some slight alterations of the oocyte organelles detected at the ultrastructural level. However, the quality of blastocysts produced from vitrified/warmed oocytes was comparable to those of blastocysts developed from fresh oocytes.

Ethical approval

The conducted research is not related to either human or animal use.

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Conflict of interest

The authors declare they have no conflict of interest.

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