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Evaluation of chromosome organization and microtubule arrangement in goat (*Capra aegragrus*) oocytes after vitrification, *in vitro* maturation and fertilization, and early embryo development

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ABSTRACT

Objective: Evaluate the use of Ethylene Glycol (EG), Dimethyl Sulfoxide (DMSO), Sucrose and Fetal Bovine Serum (FBS) as cryoprotectants and their effect on the organization of chromosomes and the arrangement of microtubules, during the vitrification process in goat oocytes matured *in vitro* and in the development of preimplantation embryos produced *in vitro*.

Design/methodology/approach: *In vitro* matured oocytes were divided into 3 groups (control group, cryoprotectant exposed group, vitrified group). A mixture of 15% EG, 15% DMSO, 0.4 M sucrose and 20% FBS was used for the vitrification using the Cryotop device. *In vitro* matured oocytes were warmed and afterwards each group was divided into two more groups. Both groups were subjected to immunofluorescence, the first group to observe the damage produced to the chromosomes and microtubules and the second group to observe the effect on the *in vitro* embryo development.

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Results: The combined use of 15% EG, 15% DMSO, 0.4 M Sucrose and 20% FBS during vitrification did not prevent cryoinjuries in goat oocytes and *in vitro* produced embryos, since embryo development was disrupted before the blastocyst stage by stopping cleavage at the morula stage. This disruption was associated with chromosome decondensation and the absence of a microtubule network, thereby hindering chromosomal segregation.
Limitations on study/implications: The effect of conventional cryoprotectants on chromosomes and microtubules arrangement on vitrified goat oocytes and *in vitro* embryo production.
Findings/conclusions: The combined use of 15% EG, 15% DMSO, 0.4 M sucrose and 20% FBS as vitrification cryoprotectants did not prevent cryoinjuries in caprine oocytes and did not improve caprine embryo development *in vitro*.

Keywords: Caprine, cryopreservation, *in vitro* embryo production, microtubules, oocytes.

INTRODUCTION

Oocyte cryopreservation is an artificial reproductive technology widely used to preserve fertility in different species. In addition, oocyte cryopreservation can be used for the preservation of species in danger of extinction and the improvement of species and breeds intended for meat, milk or wool production. Vitrification, can be used to preserve gametes and embryos at different stages of maturation, causing less damage to the cells, compared to freezing, due to the formation of ice crystals that have been correlated with a decrease in the fertilization rate, which could affect the development of the embryo and its implantation (Yurchuk *et al.*, 2018). The vitrification method has different levels of success among oocytes of different species. In fact, the oocytes show different tolerances to the cryoprotectants used in the vitrification procedures (Sudiman *et al.*, 2019). With regard to oocytes, the most reported alterations during cryopreservation are interruptions of the tubulin network, meiotic spindle disorganization, abnormal chromosomal distributions and a reduced *in vitro* fertilization rate (Tamura *et al.*, 2013, Lei *et al.*, 2014, Serra *et al.*, 2020), probably caused by the increase in aneuploidy (Buderatska *et al.*, 2020, Dviri *et al.*, 2021), genomic alterations that entail structural (Gao *et al.*, 2018, Wasielak-Politowska *et al.*, 2022) and biochemical changes (Ren *et al.*, 2019, Tsuiko *et al.*, 2019) that compromise cell viability even to the point of cell death (Balboula *et al.*, 2020, Vining *et al.*, 2021).

The composition of cryopreservation solutions plays a crucial role in the outcome of cell preservation between different species. Notably, goat oocytes are particularly susceptible to cell damage during vitrification (Youm *et al.*, 2014, Marques *et al.*, 2018). At present, there is no foolproof mix of successful chemicals for vitrification and there is still a need to clarify the optimal cryoprotective composition for goat oocyte preservation. Some compounds such as Dimethyl Sulfoxide (DMSO) and Fetal Bovine Serum (FBS) are regularly used in preservation media (Moawad *et al.*, 2012, Guo *et al.*, 2017) in different species (Fernández-Reyes *et al.*, 2012, Arcarons *et al.*, 2016), however, the proportion of these varies greatly in the literature, some of them associate it with toxicity at high concentrations (Awan *et al.*, 2020). The objective of the present study was to evaluate the effect of the combined use of Ethylene Glycol, DMSO, Sucrose and FBS to goat oocytes matured *in vitro* and to preimplantation embryos developed *in vitro*, concerning the organization of chromosomes and the arrangement of microtubules, during the vitrification process with the Cryotop device.

MATERIALS AND METHODS

Goat oocyte collection and in vitro maturation

Goat ovaries were obtained from a meat processing plant, from adult females. The ovaries were transported to the laboratory at 35 °C in isotonic saline solution (0.9% NaCl) supplemented with 100 IU/mL Penicillin and 0.1 mg Streptomycin (Sigma Aldrich, St. Louis, MO, USA). After 2 hours of transport, the ovaries were washed with isotonic saline solution and the cumulus-oocyte complexes (COC) were recovered from the ovarian follicles by follicular puncture using the technique reported by Chaves *et al.* in 2017, follicles with diameters between 2 and 8 mm were aspirated and then deposited in drops of saline physiological solution to select only COCs with uniform cytoplasm and more than 3 layers of cumulus cells, which were deposited in the *in vitro* maturation medium.

Groups of 30-35 COC were incubated in Petri dishes with drops of 100 μL of maturation medium (TCM (Tissue Culture Medium, In vitro S.A., México), supplemented with 0.3% BSA (Albumin Bovine Serum, Sigma Aldrich, USA), 5 μL/mL Follicle Stimulating Hormone (FSH; Sigma Aldrich, USA), 5 μL/mL Luteinizing Hormone (LH, Sigma Aldrich, USA), 0.32 mM Pyruvic Acid (Sigma Aldrich, USA), 10 μL/mL Penicillin-Streptomycin (P/E; Sigma Aldrich, USA)) (Izquierdo *et al.*, 2002) and then covered with mineral oil (Sigma Aldrich, USA). The samples were incubated at 38.5 °C in an atmosphere of 5% CO_2 in 95% air, with 95% humidity, for a total time of 27 h.

Oocytes vitrification and warming

Vitrification of *in vitro* cultured oocytes was performed by using a Cryotop device (Kuwayama *et al.*, 2005; Kuwayama, 2007; Liang *et al.*, 2012). First, oocytes were randomly divided into three groups: a) control (untreated oocytes), b) exposed (non-vitrified oocytes in cryoprotectant medium) and c) vitrified (vitrified cryoprotected oocytes). Exposed and vitrified oocytes were washed with 500 μL TL- HEPES buffer (In vitro S.A, México) and transferred into 300 μL holding medium (TCM-199 with Hepes; In Vitro S.A, México) supplemented with 20% FBS (38 °C for 5 min) (Gibco, USA) (Morató *et al.*, 2008; Purohit *et al.*, 2012). Next, groups of four oocytes were placed into droplets with 5 μL of equilibrated solution, a mixture of holding medium and 7.5% EG (Sigma Aldrich, USA), 7.5% DMSO (Sigma Aldrich, USA), at 38 °C for 9 min. Finally, oocytes were added immediately to the vitrification solution consisting of holding medium with 15% EG, 15% DMSO and 0.4 M sucrose (Sigma Aldrich, USA) for 1 min at 38 °C (Begin *et al.*, 2003; Morató & Mogas, 2014).

Only the vitrified group of oocytes was plunged into the liquid nitrogen (during 2 h). For warming, the tip of the Cryotop device was submerged in 3 mL of base medium supplemented with 0.5 M sucrose for 1 min at 38 °C. Then, the oocytes were recovered and washed for 3 min in four well dishes containing a 0.3, 0.25 and 0.125 M sucrose, to remove the cryoprotectants, followed by washing oocytes with holding medium and then incubated at 38 °C to complete 27 h of total incubation in maturation medium (Morató & Mogas, 2014).

In vitro fertilization and embryo development analysis

Before fertilization, 20 to 30 oocytes were washed and incubated with 100 μL of *in vitro* fertilization medium (Vitrogen, Brazil). Frozen-thawed buck semen in 0.25 mL straws was used in the experiment. A higher concentration of motile spermatozoa was obtained using a discontinuous Percoll (Sigma Aldrich, USA) density gradient (45:90). The centrifugation of semen was carried out for 20 min at 360 g, and viable spermatozoa were located primarily in the sperm pellet at the bottom of the gradient. For capacitation, the motile spermatozoa were suspended in fertilization medium to achieve a final concentration of (1×10^6 cells/mL) and incubated at 38.5 °C with 95% humidity and 5% CO_2 in air for 30 min. Both, spermatozoa and COC were incubated with fertilization medium for 18 h at 38.5 °C (Albarracín *et al.*, 2005).

After fertilization (Day 0), the presumptive zygotes were washed with PBS and denuded by gentle pipetting with embryo culture medium (Vitrogen, Cravinhos, SP, Brazil). Then, a maximum of 10 embryos were incubated in a drop with 10 μl embryo culture medium, 5 drops per dish and covered with 3 mL mineral oil for 7 d at 38.5 °C with 95% humidity and an atmosphere of 5% CO_2 in air. Cleavage stage rate was evaluated at 48 h post-fertilization, embryos with more than 6 cells were evaluated at 72 h post-fertilization, and the morulae stage rate was evaluated at 96 h, blastocyst stage rate was evaluated on days 5 to 7 post fertilization.

Oocyte immunostaining

The COC were disaggregated by pipetting in order to obtain the oocytes. Then, the oocytes were fixed with 2% paraformaldehyde for 72 h, permeabilized with 1% Triton X-100 (v/v) in PBS at 37 °C for 25 min and blocked with 1% BSA at room temperature for 15 min. Next the oocytes were incubated with anti-αtubulin coupled to Alexa-Fluor 488 (dilution 1:250; Thermo-Fisher, USA) at 4 °C overnight. Afterwards, the oocytes were washed three times with PBS and stained with DAPI (Sigma Aldrich, USA). Finally, 10 oocytes were placed on poly-L-lysine-treated slides and preserved with PBS:Glycerol (1:3). The images were obtained by a confocal microscope (Leica SP8, Wetzlar, Germany) at 63X magnification. The chromosome and microtubule criteria were based on the previous report by Albarracín *et al.* (2005). Briefly, normal spindle morphology was considered symmetrical, barrel-shaped, lacking astral microtubules and a metaphase plate diameter longer than the pole-pole distance. Spindle structure was regarded as disorganized if there was microtubule disruption, partial or total disorganization, while the absent classification equated to a complete lack of microtubules. For chromosomes, the standard organization was classified as chromosomes arranged on a compact metaphase plate at the equator of the structure. Chromosomes were classified as dispersed if they were disorganized (chromosomes misaligned at the metaphase plate) aberrant if not structured as a standard chromosome, and decondensed if a prominent less condensed appearance was observed (Albarracín *et al.*, 2005).

Statistical analysis

A two-way analysis of variance (ANOVA) was used to examine differences in chromosomes and microtubule classifications. One-way ANOVA was used to analyze differences for all the remaining variables. The ANOVA analysis was followed by a Bonferroni test for differences between means. The data are presented as mean ± standard error. Statistical significance was set at $\alpha=0.01$. All data analyses were performed using the GraphPad Software, version 6.

RESULTS

A total of 304 oocytes were evaluated for immunostaining. Both, the chromosomal organization and the spindle morphology were observed in all of the groups.

Chromosomal organization and microtubule arrangement in goat oocytes after vitrification

The chromosomes and microtubules were well organized in the control group forming a well-defined meiotic spindle; however, they were disorganized in both the exposed and Cryotop vitrified oocytes ($p<0.01$) (Figure 1A. Representative micrographs). The evaluation of *in vitro* matured oocytes presenting different chromosomal organization patterns showed 49.8% with normal chromosomes, 33.15% with dispersed chromosomes and 17.01% with decondensed chromosomes in the control group, this distribution was similar for exposed oocytes (51.09%, 28.47% and 20.43% with normal, dispersed and decondensed chromosomes, respectively) with no statistical differences compared to the control group. Vitrified oocytes did not show a statistical difference in the numbers of normal and dispersed chromosomes (39.6% and 23.99%, respectively) but had a significant increase of 42.36% decondensed chromosomes ($p<0.01$) (Figure 1B. Percentage of normal, dispersed and decondensed chromosomes). The evaluation of microtubule organization of the *in vitro* matured oocytes after vitrification resulted in 45.2% with the typical microtubule network, 33.59% with disorganized microtubules and 21.17% with absent microtubules in the control group. Exposed oocytes had no statistical difference compared to the control group, *i.e.*, 26.15% normal microtubules (and 34.73% disorganized microtubules but the percentage of absent microtubules was statistically different (39.12%) ($p<0.01$). The vitrified oocyte group had 20.99% normal microtubules, significantly lower than the control group ($p<0.01$), and 46.22% with absent microtubules, which was also statistically different from the control group. Notably, there were no statistical differences concerning percentages of disorganized microtubules for any groups (Figure 1C. Percentages of normal, disorganized and absent microtubule oocytes).

Embryo development after goat vitrified oocyte fertilization

The embryo development 48-h post-fertilization was followed (Figure 2. Embryo development). The cleavage in oocytes was significantly reduced ($p<0.01$) from 54.7% in the control group to 36.6% in exposed and to 21.3% in vitrified oocytes (Figure 2B. Cleavage stage). In addition, the +6 cells stage was also reduced from 36.3% in the control group

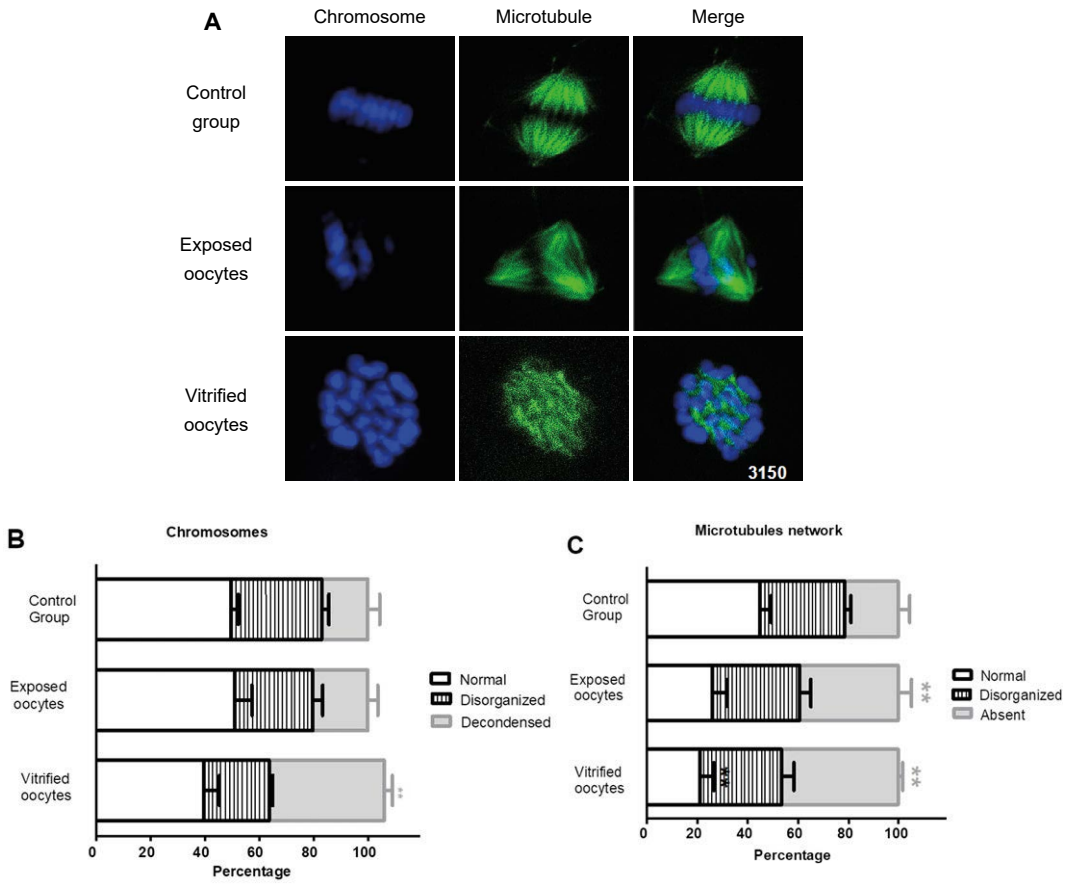

Figure 1. Oocyte vitrification increases metaphase plate disorganization and disrupts microtubule formation. A) Representative micrographs of the evaluated oocytes of the control and vitrified group, the DNA of the chromosomes are stained with DAPI (blue color) and beta-tubulin in the microtubules (green color). A) shows the barrel-shaped formation without the presence of chromosomes in any microtubule, a pattern that was found in a higher percentage in the control group (A1). While different patterns were found in the vitrified oocytes, they show an erratic arrangement (A2) and scattered decondensed chromosomes as seen in the last panel (A3). B) Percentage of normal chromosomes, dispersed chromosomes and decondensed chromosomes of each group. C) Percentages of normal, disorganized and absent microtubule oocytes in the group. Data show media ± standard error of three independent experiments. **p<0.01 *vs.* untreated oocytes.

to 11.2% and 6.6% in non-vitrified cryoprotected oocytes, respectively (Figure 2C. +6 cells stage) The morulae stage was also affected, and it was reduced from 19.2% in control group to 0.7% and 0.1% in exposed and vitrified oocytes, respectively (Figure 2D. Morulae stage) The blastocyst stage was only achieved in the control group, since no embryos were observed at this stage using exposed and vitrified oocytes (Figure 2E. Blastocyst stage).

Discussion

The rationale for animal gamete cryopreservation lies not only in preventing the extinction of many different species, but other motivations are also relevant including economic reasons, cultural identity, social role, environmental importance and scientific purposes for the benefit of humans (Kukovics, 2016). The scientific literature demonstrates a higher degree of knowledge in oocyte cryopreservation for livestock such as cow, pigs,

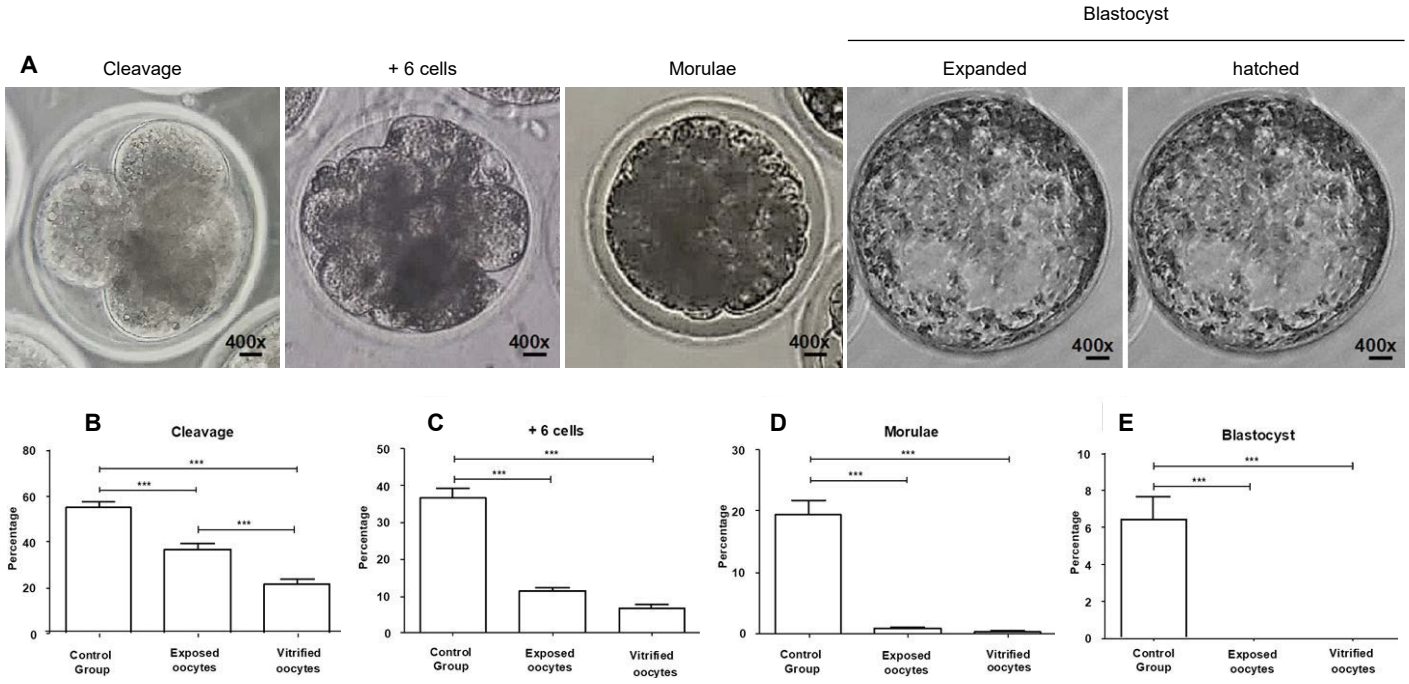

Figure 2. Embryo development was affected in exposed oocytes and vitrified oocytes. A) Representative images of embryo stages after *in vitro* fertilization by inverted bright field microscopy. Embryo development at B) cleavage stage (48 h post-fertilization), C) +6 cells stage (72 h post-fertilization), D) morulae stage (96 h post-fertilization) and E) blastocyst stage (5 days post fertilization) as quantified in the control group, exposed and vitrified goat oocytes. Data show media ± standard error of three independent experiments. ***$p<0.01$ *vs.* control group.

sheep, and buffalo than for goat oocytes (Casillas *et al.*, 2015; Kukovics., 2016, Chaves *et al.*, 2017).

In general, the failure in oocyte cryopreservation is related to the injury caused by cryoprotectants, with DMSO being one of them. In terms of cryopreservation, the reduction of DMSO has already been assessed. The use of 16% DMSO has been tested in other oocytes such as porcine oocytes (Casillas *et al.*, 2015) and 15% in bovine oocytes (Arcarons *et al.*, 2016) while the usage of 20% DMSO commonly used during cryopreservation, has not been successful for goat embryo development (Begin *et al.*, 2003; Srirattana *et al.*, 2013, Quan *et al.*, 2014). Based on this, the DMSO was decreased to 15% but unfortunately, did not improve the rate of goat embryo development.

We also tested the 20% FBS addition to the cryoprotectant mixture since proteins contained in the FBS confer cell protection. Indeed, some other studies in livestock such as pigs showed that 20% FBS was successfully used in the cryoprotectant mixture (Fernández-Reyes *et al.*, 2012) but as demonstrated in this study, no benefit was found concerning goat oocyte cryopreservation.

The causes of goat embryo development failure during cryopreservation and vitrification have not been thoroughly investigated, but the alterations in chromosome segregation and microtubule networks are reported for human oocytes during cryopreservation (Bromfield *et al.*, 2009). There is also evidence of cytoskeleton degeneration of cattle oocytes cryopreserved by liquid nitrogen (Guo *et al.*, 2017). However, literature related to goat oocyte cryopreservation has not found a highly successful cryopreservation method.

Perhaps a description of cellular mechanism of embryo development failure during the current cryopreservation methods is needed first. We demonstrated that the use of 15% DMSO and the addition of 20% FBS did not improve embryo development, and this effect was associated with chromosomal defects and microtubule network disruption. Although, the result is not disappointing either, according to Wu *et al.*, (2020) the use of 35% EG and 20% FBS, can result in a good cleaved embryo percentage, *i.e.*, 64.9% of cleaved embryos was relatively high. The two variables mentioned above, are crucial for embryo development. As it has been demonstrated, the oocyte meiotic spindle is responsible for chromosome segregation and this spindle depends on the microtubule network, and together they are a predictive marker of blastocyst ploidy in humans (Tilia *et al.*, 2020).

In summary, the use of EG, the decreased percentage of DMSO and the addition of FBS did not improve the cryoprotectant mixture used for vitrification. Indeed, this modification probably was insufficient for preventing the ice-crystal formation during the subzero reached temperatures, which cause cryoinjuries (Yurchuk *et al.*, 2018).

Regarding the cryoprotectant composition, an alternative to test in future research is DMSO between 5% and 10% and such a concentration decrease might improve embryo development. Another alternative is to replace 20% FBS with lyophilized albumin between 0.4% and 4% as has previously been tested in goat oocytes (Purohit *et al.*, 2012).

CONCLUSIONS

The cryoprotectants and the cryopreservation by vitrification methods used in this study disrupted the goat embryo development. This effect was associated with disruption of DNA integrity, as was seen by alterations in chromosome structure and also, by disruption in the microtubule network. However, vitrified cryoprotected oocytes exhibited a higher degree of alterations in terms of oocyte cleavage, +6 cells stage at 72 h post-fertilization, decondensation of chromosomes and absence of a microtubule network compared with exposed oocytes.

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