



Cat ovarian follicle ultrastructure after cryopreservation with ethylene glycol and dimethyl sulfoxide

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ABSTRACT

Ovarian tissue cryopreservation is a promising technique for fertility maintenance. The aim of this study was to compare the morphology of domestic cat ovarian follicles after tissue cryopreservation with ethylene glycol (EG) and dimethyl sulfoxide (Me₂SO). Ovaries from healthy adult cats undergoing elective ovariohysterectomy were used. Eight fragments were obtained from each pair of ovaries: two were used as fresh controls; three were submitted to fresh perfusion toxicity test and perfused with M199, 10% fetal calf serum and 0.4% sucrose containing Me₂SO 1.5 M, EG 1.5 M or Me₂SO 0.75 M + EG 0.75 M; and the remaining three fragments were perfused as described and submitted to slow freezing. After 45 days of cryopreservation, the samples were thawed, fixed and processed for light and transmission electron microscopy (TEM). The percentages of morphologically normal follicles identified by light microscopy were higher in the control group (94.45%) in comparison to the frozen groups (80.56% with EG, 78.7% with Me₂SO and 75.87% with EG + Me₂SO). The fresh perfused tissue showed no statistical difference compared to control or frozen samples. The TEM analysis showed less damage in the ultrastructure of follicles from the Me₂SO group in comparison with the EG and Me₂SO + EG groups. According to the morphological analysis, 1.5 M Me₂SO is the best cryoprotectant for cryopreservation of domestic cat ovarian tissue regarding the morphology of preantral follicles after thawing. Further studies regarding the viability of these follicles should be performed.

1. Introduction

Reproductive biotechnology has been widely developed for reproduction of valuable animals, women who need to undergo cancer therapy and for the conservation of endangered species [29]. Cryopreservation is an important technology to maintain cell viability during long-term storage and has been widely applied in female reproductive tissues and cells [6,9,22,23,26], allowing the development of germplasm banks and preservation of genetic variability [17].

As most felid species are included in the International Union for the Conservation of Nature (IUCN) Red List of endangered species [14], there is worldwide concern about their preservation. Allied to efforts in maintaining preserved habitat areas, the development of new assisted reproduction techniques is essential to explore genetic sources from wild species [17]. Access to wild felid reproductive tissues for experimental purposes, however, is extremely restricted. Therefore, the

domestic cat becomes a valuable experimental model for developing reproductive techniques to be applied in wild felids [17].

Two approaches may be considered for female germ cell storage: cryopreservation of oocytes obtained from antral follicles or ovarian tissue samples containing many preantral follicles. In fact, ovarian tissue has already been cryopreserved in several species (for review, see Refs. [1,16,29]). Moreover, transplantation of cryopreserved ovarian tissue has demonstrated function restoration in animals [11,33,34] and more than 130 live births in humans [10,15,25]. Additionally, the possibility of collecting ovarian tissue from any developmental age (from fetal stages to adult age) and individual status (alive or dead) is a remarkable advantage for the preservation of wild animals (reviewed by Santos et al. [29]).

Some attempts have been made to cryopreserve wild felids [35,36] and domestic cat ovarian tissue [4,5,18,21,24,27,32]. However, the protocols have not been well established to date. Thus, the aim of this

Abbreviations: CPA, cryoprotectant agent; EG, ethylene glycol; Me₂SO, dimethyl sulfoxide; MN, morphologically normal; TEM, transmission electron microscopy

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study was to compare three cat ovarian tissue slow freezing protocols using: ethylene glycol (EG), dimethyl sulfoxide (Me₂SO) and an association of both cryoprotectant agents (CPA).

2. Material and methods

2.1. Animals and ovary collection

Unless individually specified, the reagents were obtained from Sigma Aldrich (Sigma Chemical Co., St Louis, MO, USA).

The ovaries of nine mixed breed healthy adult queens (*Felis catus*) were used. Animals ranging from 1 to 3 years old were submitted to elective ovariohysterectomy procedure in a veterinary clinic (Empório dos Bichos, Brasília, Brazil). Only macroscopically normal ovaries with no corpus luteum or cysts were used. Due to the cyclicity of the animals, antral follicles were commonly seen on the ovaries.

All nine pairs of ovaries were transported to the laboratory in 0.9% saline solution at 36 °C within 1 h. The adipose tissue and ligaments were excised, the ovaries were washed in 70% ethanol and 0.9% saline solution and antral follicles were sliced. Using a millimeter graph paper as template, pieces (2 mm × 2 mm × 10 mm) containing both cortex and medulla parts of the ovary were cut with a scalpel blade, in a total of eight pieces from each pair of ovaries.

2.2. Cryopreservation protocol and experimental design

The experimental design was based on that described by Borges et al. [3]. Two of the eight fragments obtained from each pair of ovaries were taken as fresh controls and immediately fixed for histology and transmission electron microscopy (TEM) analysis. The six remaining fragments were submitted to CPA perfusion; duplicate fragments were perfused with one of the three different CPA solutions: 1.5 M Me₂SO, 1.5 MEG or an association of 0.75 M Me₂SO + 0.75 M EG. The aforementioned CPA solutions were solubilized in M199 with 0.4% sucrose and 10% fetal calf serum (Gibco BRL, Life Technologies, Grand Island, NY, USA). For CPA perfusion, the fragments were maintained in 1 mL of the elected solution at 10 °C for 10 min.

One of the two fragments perfused with each CPA solution was immediately submitted to CPA removal by three 5-min baths in decreasing CPA concentrations (50%, 25% and no CPA). These fresh perfused tissue samples were processed only for light microscopy analysis in order to survey immediate toxic or osmotic effects caused by the CPAs on the follicles morphology.

The three remaining fragments were frozen after perfusion, according to Borges et al. [3], using a programmable freezer (Dominium K, Biocom, Brazil). Cryovials were cooled to -7 °C at -2 °C/min and maintained at this temperature during 15 min for the seeding procedure, which consisted in touching the tube with a cotton-tip spatula chilled in liquid nitrogen. The samples were then cooled to -35 °C at -0.3 °C/min and finally immersed in liquid nitrogen (-196 °C).

After 45 days storage, samples were removed from liquid nitrogen

Table 1

Percentages (Mean ± SD) of morphologically normal follicles found in cat ovarian tissue submitted to different treatments (fresh control tissue, fresh perfused tissue and frozen/thawed tissue) using three cryoprotectant solutions.

Treatment	Cryoprotectant agent	% MN Follicles (Primordial + Growing)	% MN Primordial Follicles	% MN Growing Follicles
Fresh Control		94.45 ± 4.80 ^a	95.12 ± 4.47 ^a	82.02 ± 21.33 ^a
Fresh perfused Tissue	EG	90.79 ± 3.50 ^{ab}	90.34 ± 2.55 ^{ab}	87.55 ± 18.20 ^a
	Me ₂ SO	88.80 ± 9.69 ^{ab}	88.91 ± 9.21 ^{ab}	86.32 ± 17.45 ^a
	EG + Me ₂ SO	83.11 ± 10.38 ^{ab}	82.88 ± 8.61 ^{ab}	79.48 ± 29.12 ^a
Frozen/Thawed Tissue	EG	80.56 ± 8.57 ^b	80.06 ± 10.19 ^b	74.24 ± 21.75 ^a
	Me ₂ SO	78.70 ± 11.36 ^b	78.91 ± 12.57 ^b	76.47 ± 25.65 ^a
	EG + Me ₂ SO	75.87 ± 13.89 ^b	78.85 ± 13.17 ^b	70.27 ± 26.63 ^a

^{a, b} Values showing different letters in the same column are statistically different ($p < 0.05$). MN: morphologically normal; EG: 1.5 M ethylene glycol; Me₂SO: 1.5 M dimethyl sulfoxide; EG + Me₂SO: 0.75 M of EG + 0.75 M of Me₂SO.

and subjected to the thawing protocol. Cryovials were maintained at room temperature (approximately 26 °C) for 1 min and immersed in water at 37 °C for 4 min. After complete thawing of the samples, CPA was removed as previously described. Samples from frozen/thawed treatments were fixed and processed for light microscopy and TEM.

2.3. Light microscopy analysis

Samples from all treatment groups were fixed in Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 2 h, dehydrated in increasing concentrations of ethanol, diafanized with xylene and embedded in Paraplast. The blocks were serially sectioned (5 μm thick) and from each five sections one was mounted and stained with haematoxylin and eosin for evaluation under light microscopy; on average, 60–80 sections were analyzed from each tissue sample. Preantral follicles were counted and classified as primordial (oocytes surrounded by a single layer of flattened granulosa cells) or growing follicles (follicles surrounded by one or more complete layers of cuboidal granulosa cells) and as morphologically normal (MN) or degenerated. Spherical and intact follicles, attached to surrounding stroma cells, with a uniform distribution of granulosa cells and a spherical oocyte showing no retractions or vacuoles were considered normal. Only follicles presenting a visible oocyte nucleus were counted and classified as MN or degenerated.

2.4. Transmission electron microscopy analysis

A small sample from each fresh control and frozen/thawed fragment was fixed in modified Karnovsky (2.5% glutaraldehyde, 2% paraformaldehyde in sodium cacodylate buffer 0.1 M, pH 7.2) for 4 h. Samples were subsequently washed in sodium cacodylate buffer (0.1 M), post fixed with osmium tetroxide (1%), potassium ferricyanide (1.6%) and calcium chloride (5 mM) and contrasted *in bloc* with uranyl acetate (0.5%). The samples were then rinsed and dehydrated in increasing concentrations of acetone and embedded in Spurr resin. After embedment, semi-thin sections (3 μm) were cut for location of the follicles. Thin sections (70 nm) from each follicle were analyzed in a transmission electron microscope (JEOL, JEM 1011, Japan). The organelles, basal and plasmatic membranes, together with the nuclear envelope of oocytes and granulosa cells, were analyzed. To identify early degeneration signs, not seen under lower magnifications (light microscopy), only follicles that showed normal morphology on semi-thin section were evaluated by TEM.

2.5. Statistical analysis

The percentages of MN and degenerated follicles as well as primordial and growing follicles were calculated for each sample. Data were transformed to arcsin√% and submitted to analysis of variance (ANOVA). Means were compared between treatments using the Tukey test. Differences were considered significant when $P < 0.05$.

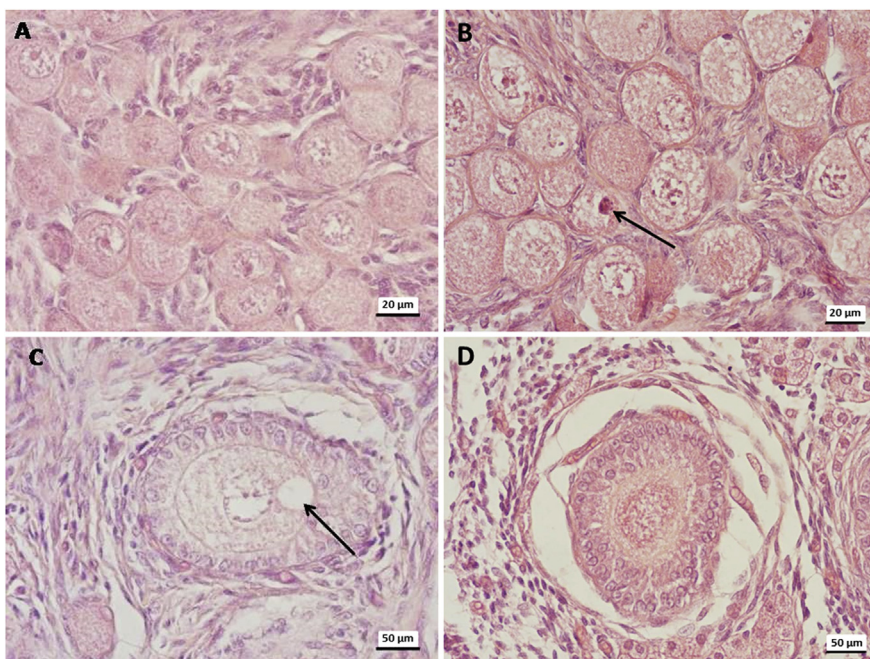


Fig. 1. Domestic cat preantral follicles stained with haematoxylin and eosin. A) pool of morphologically normal primordial follicles from fresh ovarian tissue; B) primordial follicle with pyknotic nucleus (arrow), from fresh perfused ovarian tissue; C) growing follicle with vacuole in the oocyte cytoplasm (arrow), from frozen/thawed ovarian tissue; D) growing follicle detached from stroma, from frozen/thawed ovarian tissue.

3. Results

3.1. Light microscopy

A total of 4582 follicles were evaluated by light microscopy: 3893 (84.96%) were classified as primordial and 689 (15.04%) as growing follicles. The mean percentage of MN follicles was calculated for each

treatment after light microscopy evaluation (Table 1). Statistical analysis revealed significantly lower percentages of MN primordial follicles ($P < 0.05$) in all freezing treatments in comparison to the control group. No statistical differences were observed between the fresh control and fresh perfused groups or between fresh perfused groups and frozen/thawed groups. The most frequently observed degeneration signs were: pyknotic nucleus of the oocyte in primordial follicles,

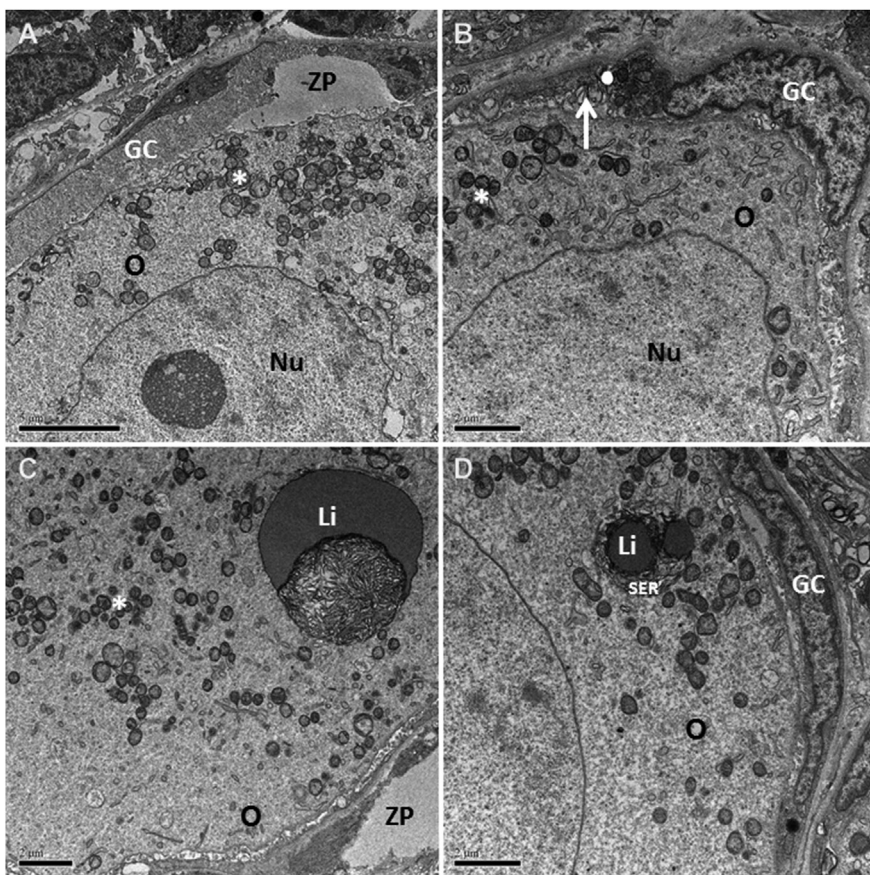


Fig. 2. Transmission electron micrographs of preantral follicles from fresh cat ovarian tissue (control). A) Onset of zona pellucida (ZP) formation and adhesion between oocyte and granulosa cell (GC). B) Microvilli between oocyte and granulosa cells (arrow); C) lipid droplet (Li) with associated endoplasmic reticulum cisterns; D) presence of smooth endoplasmic reticulum (SER) attached to the lipid droplet (Li). GC: granulosa cell, O: oocyte, Nu: oocyte nucleus, ZP: zona pellucida, SER: smooth endoplasmic reticulum, (*) mitochondria. Bars: 5 μm in A, 2 μm in B-D.

detachment from the ovarian stroma and vacuoles in the cytoplasm of the oocyte in growing follicles (Fig. 1).

3.2. Transmission electron microscopy (TEM)

On TEM evaluation, follicles ($n = 4$) from fresh control samples showed oocytes adhering to granulosa cells or early formation of zona pellucida with extensions of plasmatic membrane (Fig. 2). In the oocytes, organelles were well-distributed within the cytoplasm, with numerous rounded mitochondria (Fig. 2A, B and C) and endoplasmic reticulum cisternae (Fig. 2C and D). In addition, the follicles showed medium-sized lipid droplets with different electron densities, which were often associated with endoplasmic reticulum cisternae (Fig. 2C and D).

Samples from fresh perfused groups were not evaluated by TEM, since there were no statistic differences regarding the light microscopy analysis in comparison to the other groups.

The follicles ($n = 12$) in the ovarian tissue frozen with Me_2SO (Fig. 3) showed great similarity with the control group. Mitochondria showed normal electron density and were well-distributed (Fig. 3A and B). Few mitochondria were classified as pleomorphic and contained many ridges. Zona pellucida material was observed in some follicles between the oocyte and granulosa cells, in which microvilli projections were identified (Fig. 3A). There were also medium-sized lipid droplets usually associated to endoplasmic reticulum cisternae (Fig. 3A).

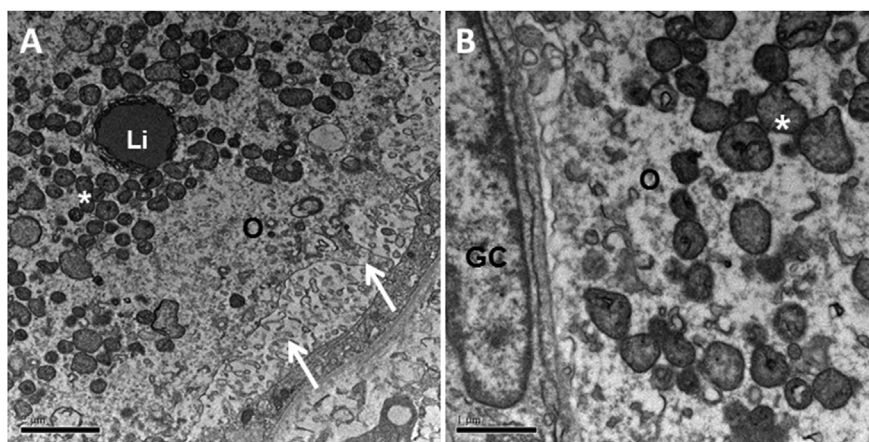


Fig. 3. Transmission electron micrographs of preantral follicles from cat ovarian tissue cryopreserved with Me_2SO . A) mitochondria (*) with normal electron density, well-distributed within the oocyte cytoplasm and a lipid droplet (Li) with surrounding smooth endoplasmic reticulum cisternae, microvilli in the zona pellucida region (white arrows); B) strong adhesion between oocyte and granulosa cell (GC), mitochondria (*). Bars: 2 μm in A and 1 μm in B.

Follicles ($n = 8$) from ovarian tissue cryopreserved with EG showed numerous ultrastructural alterations (Fig. 4). Detachment of the oocyte from granulosa cells was observed (Fig. 4A) together with granulation in the cytoplasm, dilated endoplasmic reticulum cisternae and clear vesicles (Fig. 4A). Furthermore, the area surrounding the oocyte nucleus presented few or no organelles (Fig. 4B). Large lipid droplets were observed in the oocyte cytoplasm of some of the follicles (Fig. 4B).

Follicles ($n = 5$) from ovarian tissue cryopreserved with EG + Me_2SO also showed clear signs of degeneration (Fig. 5), with detachment of oocyte and granulosa cells (Fig. 5A, B and C). The oocyte cytoplasm had a clotted appearance (Fig. 5C) and organelles were not evenly distributed throughout the cytoplasm (Fig. 5D). Moreover, disruption of the plasmatic membrane was observed at various points (Fig. 5C) together with separation of the inner and outer layers of the nuclear envelope (Fig. 5D).

4. Discussion

In the present study, we compared the effects of EG, Me_2SO and a combination of EG/ Me_2SO in the slow freezing of domestic cat ovarian tissue. After freezing/thawing, light microscopy revealed no significant difference between the three CPA solutions regarding the percentage of

MN follicles (75–80%). TEM analyses showed that Me_2SO was the most suitable CPA for the slow freezing of cat ovarian tissue based on the morphology evaluation of the follicles.

No difference between the percentage of MN follicles from fresh tissue perfused with CPAs and fresh control tissue was identified in the present study. On the contrary, the reduction of MN follicles was observed in a similar perfusion test performed in bovine ovarian tissue using EG in comparison to Me_2SO [20], proving the toxic effects of EG in this species even without freezing. These results show that tissues from different species react differently to the same CPA, highlighting the importance of species-specific slow freezing protocols. The toxic effects of EG have also been demonstrated in endothelial cells [7], and may be summarized by changes in signaling pathways, glycoproteins, and even in genes involved in extracellular and transmembrane functions.

We observed 80% of MN follicles when using EG. Other studies using the same CPA obtained considerably lower percentages of MN follicles for cat and wild felid ovarian tissue. Lima et al. [18] compared EG and glycerol for cat ovarian tissue slow freezing and found a lower percentage of MN follicles (58%) with EG in comparison to our results. In wild felids, the use of EG as CPA for slow freezing of ovarian tissue resulted in a significant reduction of normal primordial follicle population to 31–57% [36]. Discrepancies in the results may lie in the different freezing curve and basic medium used. Moreover, the low percentages of MN follicles reported after using EG as a CPA for cat ovarian

tissue in many studies indicate that this is not the most suitable for this species.

Our TEM analysis data corroborates this conclusion, although a high percentage of MN follicles were seen in a first analysis. Preantral follicles in ovarian tissue cryopreserved with EG presented several ultrastructural alterations, such as an organelle-free zone in the perinuclear region, detachment of oocytes from granulosa cells, plasmatic membrane disruption and vesicles in the oocyte cytoplasm. Analysis by TEM allows the observation of very early degeneration signs, which would probably compromise further development of the cells. Rodrigues et al. [28] also observed vacuolization in the oocyte cytoplasm and loss of granulosa cell content in preantral follicles when EG was used as the CPA for goat ovarian tissue cryopreservation.

In the present study, the association of Me_2SO and EG was the most deleterious treatment for slow freezing of cat ovarian tissue. Although the combination of these CPAs resulted in 75.87% of MN follicles under light microscopy, TEM analysis showed many ultrastructural alterations. The association of Me_2SO and EG was used for cat ovarian tissue vitrification [24] and demonstrated 67.5% of MN follicles after warming. The association of these two CPAs also resulted in good structural integrity of follicles after vitrification of mice ovarian tissue [12]. Vitrification protocols demand a much higher concentration of

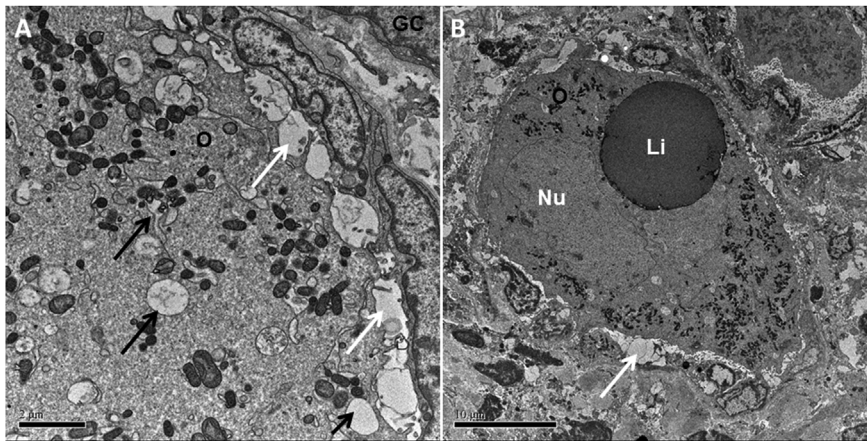


Fig. 4. Transmission electron micrographs of preantral follicles from cat ovarian tissue cryopreserved with EG. A) Follicle showing oocyte (O) detachment from granulosa cells (GC) (white arrows) and numerous clear vesicles (black arrows) in the oocyte cytoplasm; B) A large lipid droplet (Li) pressing the oocyte nucleus (Nu), note the space between the oocyte and granulosa cells (white arrow). Bars: 2 μm in A and 10 μm in B.

CPA, and the association of two or more CPAs may minimize the deleterious effects of each, by reducing their concentration, while a combination affords the viscosity required to vitrify the sample [2]. In our slow freezing protocol, the combination of two CPAs may have resulted in a concentration of each constituent that was too low to induce a protective effect. Regarding Me_2SO , it is known that its effects in membranes and, consequently, in cell dehydration, depend on its concentration: when lower than 10% (approximately 1.5 M), the bilayer conformation of cell membrane is maintained and few pores are formed, diminishing the water output from cells and increasing the chances of ice crystal formation; when the concentration of Me_2SO is between 10 and 20%, it has the ability to form hydrophilic pores in the cell membrane, allowing cell dehydration; when the concentration is above 20%, it destroys the integrity of the lipid bilayer [8]. The separation of inner and outer layers of plasmatic membrane observed by

TEM analysis may have occurred because of ice crystal formation within the cells followed by destabilization of the membranes after thawing, probably caused by low CPA concentrations. This suggests that for cat ovarian tissue cryopreservation, CPA association demands a minimum concentration of each cryoprotectant to be effective.

Me_2SO toxicity, in turn, may be related to protein aggregation in cells, being less deleterious than other CPAs [13]. Using 1.5 M Me_2SO , we observed 83.11% of MN follicles after thawing. Tanpradit et al. [32] used the same concentration of Me_2SO during cat ovarian tissue slow freezing and observed 47.4% of MN follicles in post-thawed tissue. Differences between these results and ours may lie in the different cooling rate. In contrast, a percentage of MN follicles similar to the present study (84%) was found after slow freezing of dog ovarian tissue with the same Me_2SO concentration [19]. In the present study, the ultrastructural evaluation showed that ovarian cryopreservation with

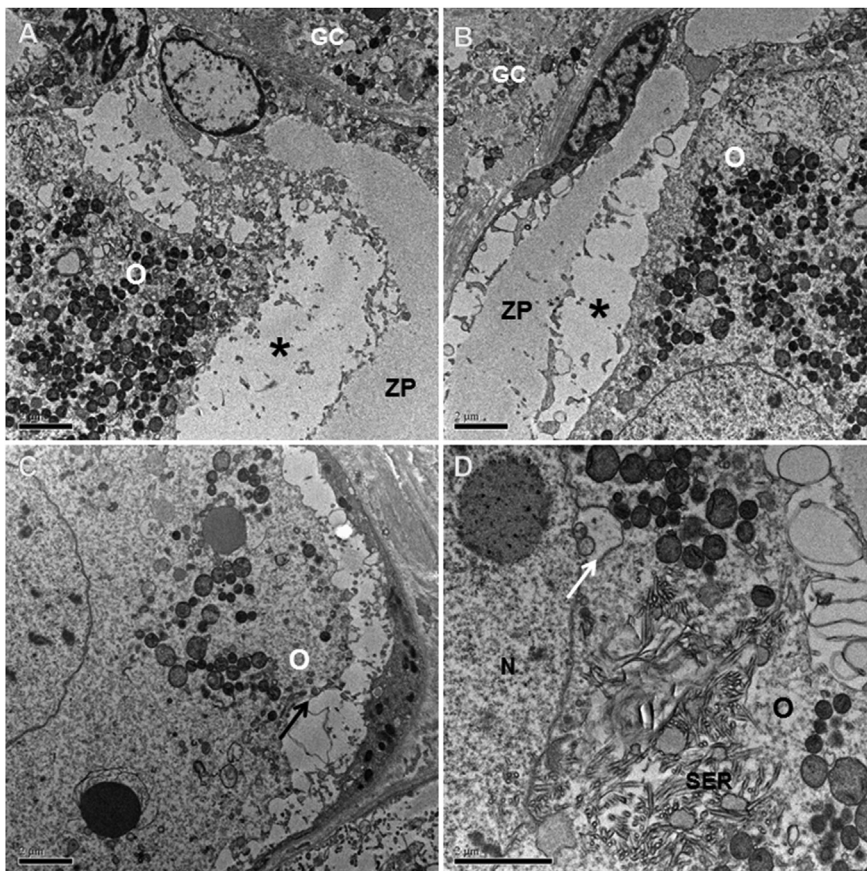


Fig. 5. Transmission electron micrographs of preantral follicles from cat ovarian tissue cryopreserved with EG + Me_2SO . A and B) Degenerated follicles showing detachment (*) of zona pellucida (ZP) from the oocyte (O) and the granulosa cells (GC). C) Oocyte with disruption of the plasmatic membrane (black arrow); also note the clotted appearance of the ooplasm. D) Separation of the inner and outer layers of the nuclear envelope (white arrow) and disorganization of the smooth endoplasmic reticulum (SER). N: nucleus. Bars = 2 μm .

Me₂SO maintained follicle morphological integrity. After comparison with other CPAs, Me₂SO was also shown to be the most suitable for slow freezing of cow [20], bitch [19] and sow [3] ovarian tissue.

The present study aimed to determine the best CPA for feline ovarian tissue slow freezing. According to histological evaluation, the three protocols used showed similar results. However, TEM revealed a higher efficiency of 1.5 M Me₂SO in maintaining the ultrastructure of the follicle complex. Further studies are needed regarding the ability of these follicles to grow after freezing/thawing.

Declaration of interest

All authors declared that they do not have any potential conflict of interest.

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