Cryopreservation of swine ovarian tissue: Effect of different cryoprotectants on the structural preservation of preantral follicle oocytes

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Abstract

The present study aimed to test different cryoprotectants on cryopreservation of pig ovarian tissue. Pig ovaries (n = 3) were collected at a local slaughterhouse. From each ovary, ten cortex samples were taken. One was immediately fixed (control) and another placed in short-term tissue incubation (STTI control). The other 8 samples were cryopreserved, in pairs, using 4 different cryoprotectants: dimethyl sulphoxide (Me2SO – 1.5 M), ethylene glycol (EG – 1.5 M), propanediol (PROH – 1.5 M) and glycerol (GLY – 10%), all with 0.4% sucrose. Samples were slow cooled and stored in liquid nitrogen for 7 days. After thawing and cryoprotectant removal, one sample from each treatment was immediately fixed and the other was placed in short-term tissue incubation (STTI) for 2 h and then fixed. Samples were processed for histology and transmission electron microscopy. The percentages of morphologically normal follicles (MNF) in cryopreserved tissue using Me2SO (67.0 ± 4.9), EG (81.8 ± 1.4) and PROH (55.9 ± 9.9) were significantly lower ($P < 0.05$) than observed in fresh control tissue (97.7 ± 1.2). When ovarian tissue was cryopreserved with GLY, no morphologically normal follicles could be found (0%). After STTI, PROH showed a significantly lower percentage of MNF when compared with all other treatments and the control. After ultrastructural analysis, follicles cryopreserved with Me2SO and EG showed some small alterations, but no signs of advanced degeneration. Overall, these were similar to follicles from the control group. In conclusion, it is possible to cryopreserve preantral follicles from pig ovarian tissue using Me2SO or EG.

Keywords: Pig; Me2SO; Ethylene glycol; Morphology; Ultrastructure

Introduction

The use of oocytes in reproductive techniques may offer means of improving germplasm banks, as well as propagating valuable animal stocks and endangered species. Oocyte cryopreservation has frequently been attempted, but consistently with poor results. Among the oocytes of mammalian species, those of pigs appear to be extremely sensitive to low temperatures [7]. Therefore, as yet there have been no reports of successful cryopreservation of porcine oocytes by traditional slow freezing [30]. Detrimental effects of cooling on porcine oocytes were observed after IVM, and included reduced normal spindle formation, and decreased nuclear and cytoplasmic maturation [15].

An alternative strategy for storing female germ cells is the cryopreservation of ovarian tissue. This method enables the storage of large numbers of oocytes (within preantral follicles). Unlike fully-grown oocytes, oocytes in preantral follicles seem to tolerate cryopreservation well [10] and [27]. Oocytes within preantral follicles have several characteristics that should make them less vulnerable to cryoinjury than mature oocytes. The most important of these characteristics are: (a) the small size of the oocyte and its support cells; (b) its low metabolic rate; (c) the cell cycle stage (arrested at prophase of meiosis I); (d) the absence of a zona pellucida and lack of peripheral cortical granules; and (e) the small amount of cold-sensitive intracytoplasmic lipid [10] and [27]. Cryopreservation of ovarian tissue offers even more advantages, because ovarian tissue collection is not dependent on age or the stage of the estrous cycle and can even be applied to animals that die unexpectedly [27].

Cryopreservation of ovarian tissue proved to be effective for a wide range of species including sheep [3], [4], [10], [20] and [24], cattle [5], [16] and [21], goats [22] and [25] and humans [18] and [26].

However, there is no information about cryopreservation of preantral follicles or ovarian tissue in pigs. The cryopreservation of immature oocytes in preantral follicles may be a viable alternative for the conservation of swine female germ cells. The aim of the present study was to evaluate the effect of different cryoprotectants on swine preantral follicles after ovarian tissue cryopreservation.

Material and methods

Three ovaries (from three different animals) were collected from gilts a local abattoir and transported to the laboratory at 36–38 °C within 1 h. In the laboratory, ovaries were trimmed and rinsed with 70% ethanol and sterile saline solution. Ten small strips of ovarian cortex (2 mm × 1 mm × 1 cm) were taken from each ovary. One piece from each ovary was chosen at random to be the control and immediately fixed, another was placed in short-term tissue incubation for 2 h (STTI control) and then fixed. The other eight pieces were randomly assigned to one of the four cryoprotectants used, two samples per treatment.

The four cryoprotectans used in this study were: dimethyl sulphoxide (Me2SO – 1.5 M), ethylene glycol (EG – 1.5 M), propanediol (PROH – 1.5 M) and glycerol (GLY – 10%). All cryoprotectant solutions were prepared in PBS with 0.4% M sucrose.

Ovarian tissue was frozen according to the method described for bovine ovaries [16]. Briefly, each ovarian sample was placed into a 1.2 ml cryogenic vial containing 1.0 ml of cryoprotectant solution, and frozen using a programmable freezer (Dominium K, Biocom, Brazil). Vials were equilibrated at 10 °C for 20 min and then cooled from 10 °C to −7 °C at 1 °C/min and held at this temperature for 10 min. At this point, samples were manually seeded and then cooled at 0.3 °C/min to −30 °C. The vials were then plunged into liquid nitrogen (−196 °C) and stored for 7 days. Samples were thawed by warming the cryovials in air for 30 s, followed by immersion in water at 38 °C until ice melted. The cryoprotectant was removed by washing the tissue samples three times (5 min each), twice in PBS containing 0.4% sucrose and decreasing concentrations of the cryoprotectants (0.5 and 0.25 times the concentration used for the cryopreservation), and once in PBS. After freezing and thawing, one of the ovarian slices from each treatment was placed into short-term tissue incubation (STTI) and a small piece was taken from the other and fixed for transmission electron microscopy (TEM). The rest was fixed for light microscopy (LM). A chart of the experimental treatments is presented in Fig. 1.

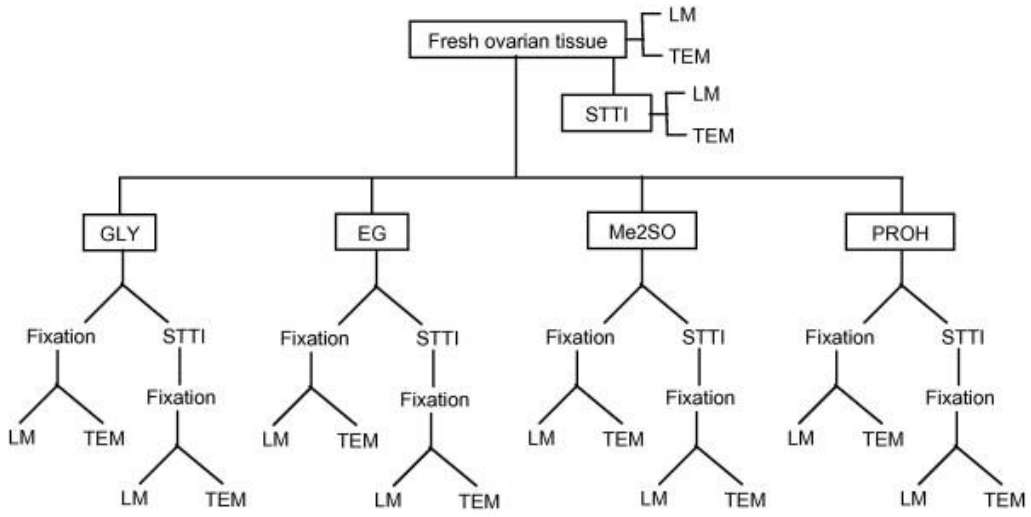

Fig. 1. Experimental protocol to test the effect of different cryoprotectants on the morphology of preantral follicles in cryopreserved swine ovarian tissue. LM – light microscopy; TEM – transmission electronic microscopy; STTI – short-term tissue incubation.

Short-term tissue incubation

For the short-term tissue incubation, each ovarian fragment was placed in 1 ml culture medium in a 4-well dish (Nunc, Roskild, Denmark) for 2 h. Dishes were kept at 38.5 °C in a humidified atmosphere of 5% CO2 in air. The culture medium consisted of Waymouth MB 752/1 supplemented with 0.23 nm pyruvic acid, 2 mM l-glutamine, 6.25 µg/ml insulin, 6.25 µg/ml transferring and 6.25 ng/ml selenium (ITS), 100 µm/ml l-ascorbic acid, 100 µg/ml

penicillin, 50 µg/ml streptomycin (all from Sigma Chemical Co., St. Louis, MO, USA) and 5% foetal calf serum (FCS) (Gibco BRL, Life Technologies, Grand Island, NY, USA). The aim of this short-term tissue incubation was only to allow the tissue to return to its normal temperature and metabolism [21], there was no intention to promote growth. The cells could therefore morphologically express molecular damage that could have occurred during cryopreservation.

Light microscopy evaluation

Samples were fixed in Carnoy fixative for 4 h. Samples were then dehydrated in ethanol, clarified with xylene, embedded in paraffin wax and sectioned at 5 µm thickness. The sections were stained with hematoxylin and eosin (HE), end examined under a light microscope (Axiophot, Zeiss, Oberkochen, Germany). Only preantral follicles with visible nuclei were counted. Preantral follicles were classified, according to their developmental stage, as early preantral (one layer of flattened or flattened-cuboidal granulosa cells around the oocyte) or growing (one or more layers of cuboidal granulose cells around the oocyte). Follicles were also classified as morphologically normal (MNF) or degenerated. Follicles were considered degenerated when presenting pycnotic bodies in granulosa cells, condensed oocyte nucleus, shrunken oocyte, oocyte cytoplasm vacuolization or low cellular density.

Transmission electron microscopy evaluation

Small pieces of ovarian cortex were fixed in Karnowisky (2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2) for 3 h at room temperature. After being washed with sodium cacodylate buffer, the ovarian pieces were postfixed in a solution containing 1% osmium tetroxid, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. Subsequently, the samples were dehydrated in acetone and embedded in Spurr. Semi-thin sections (3 µm) were stained with Toluidine Blue. Thin sections (70 nm) were stained with uranyl acetate and lead citrate, and examined in a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Only preantral follicles (n = 26) from the controls and treatments that presented higher percentages of MNF in the light microscopy evaluation and with normal morphology in semi-thin sections were evaluated for ultrastructural organization. For evaluation by transmission electron microscopy, characteristics of oocyte and granulosa cells, their organelles, basal, plasmatic and nuclear membranes were observed.

Statistical analysis

The percentages of morphologically normal follicles were compared among treatments. Data were transformed to arcsine √ and analyzed by ANOVA and Tukey test. Values were considered statistically significant when $P < 0.05$ and are presented as mean ± S.D. calculated from three replicates.

Results

A total of 708 preantral follicles were analyzed (85 on average per treatment). When ovarian tissue was cryopreserved with GLY, no morphologically normal follicle (MNF) could be found (0% MNF). The percentages of MNF in fresh and cryopreserved tissue using Me2SO, EG or PROH as cryoprotectant, both before and after short-term tissue incubation are presented in Fig. 2. A very low rate of degeneration was observed in non-cryopreserved ovarian tissue (control: 97.7% MNF, and STTI control: 97.5% MNF). In ovarian pieces cryopreseved with Me2SO, EG or PROH, a significantly lower percentage of MNF was observed when compared with the control ($P < 0.05$). In contrast, after the short-term tissue incubation (STTI), the only treatment that differed from STTI control was cryopreservation with PROH ($P < 0.05$). Before STTI, no difference was observed when comparing cryoprotectants among each other. After STTI, PROH presented significantly less MNF than Me2SO and EG ($P < 0.05$).

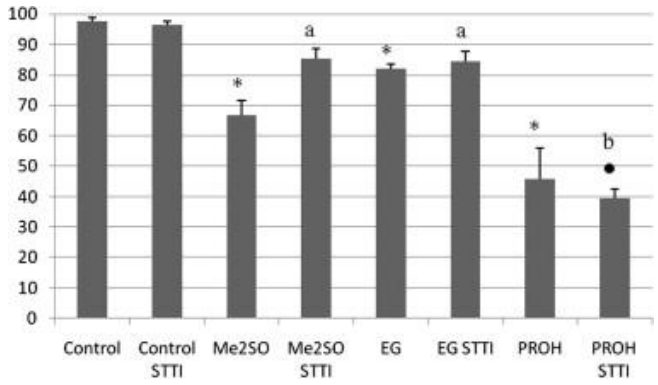

Fig. 2. Mean percentage (±S.D.) of morphologically normal preantral follicles (MNF) in pieces of fresh ovarian tissue (control) and frozen-thawed ovarian tissue using different cryoprotectants (Me2SO, EG or PROH) before and after short-term tissue incubation (STTI).
* Differ from the control; ● Differ from the STTI control ($P < 0.05$). ab Different letters indicate differences among cryoprotectants after STTI. ($P < 0.05$).

Considering only early preantral follicles (Fig. 3), significantly fewer MNF were observed in tissues cryopreserved with all three cryoprotectants when compared to the control ($P < 0.05$), and after STTI only PROH presented fewer MNF than STTI control ($P < 0.05$).

Among cryoprotectants, EG showed higher percentages of MNF than PROH before STTI, and EG and Me2SO presented higher percentages of MNF than PROH after STTI ($P < 0.05$). Moreover, when comparing the results from the same cryoprotectant before and after STTI, a significant increase in the percentage of MNF was observed for Me2SO after STTI ($P < 0.05$).

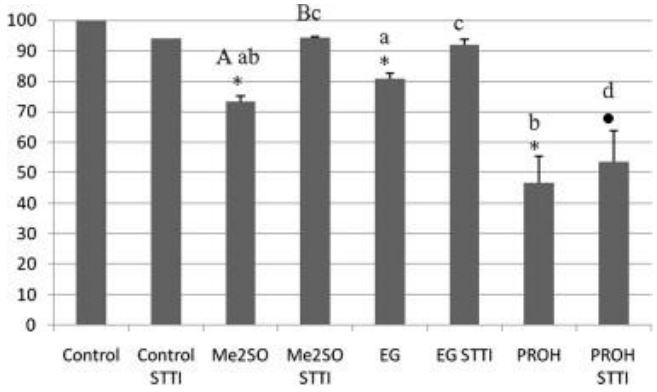

Fig. 3. Mean percentage (±S.D.) of morphologically normal early preantral follicles (MNEPF) in pieces of fresh ovarian tissue (control) and frozen-thawed ovarian tissue using different cryoprotectants (Me2SO, EG or PROH) before and after short-term tissue incubation (STTI). * Differ from the control ($P < 0.05$); ● Differ from the STTI control ($P < 0.05$). ab Different letters indicate differences among cryoprotectants before STTI ($P < 0.05$). cd Different letters indicate differences among cryoprotectants after STTI ($P < 0.05$). AB Different letters indicate differences between before and after STTI for the same cryoprotectant ($P < 0.05$).

Considering growing preantral follicles (Fig. 4), significantly fewer MNF were observed in tissues cryopreserved with Me2SO and PROH when compared to the control ($P < 0.05$), and after STTI all three cryoprotectants presented fewer MNF than STTI control ($P < 0.05$). Moreover, PROH showed significantly inferior results than EG and Me2SO both before and after STTI ($P < 0.05$).

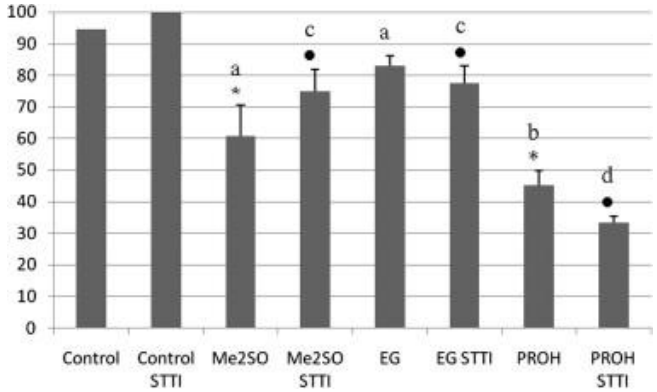

Fig. 4. Mean percentage (±S.D.) of morphologically normal growing follicles (MNGF) in pieces of fresh ovarian tissue (control) and frozen-thawed ovarian tissue using different cryoprotectants (Me2SO, EG or PROH) before and after short-term tissue incubation (STTI).
* Differ from the control ($P < 0.05$); ● Differ from the STTI control ($P < 0.05$). ab Different letters indicate differences among cryoprotectants before STTI ($P < 0.05$). cd Different letters indicate differences among cryoprotectants after STTI ($P < 0.05$).

At the histological analysis, MNF were characterized by a round or oval oocyte, presenting a well-delimited nucleus with uncondensed chromatin, surrounded by well-

organized granulosa cells without pycnotic nuclei (Fig. 5A and B). In degenerated follicles, the most predominant characteristics were picnosis of oocyte nucleus, shrunken oocyte, oocyte cytoplasm vacuolization and disorganized granulosa cells (Fig. 5C and D).

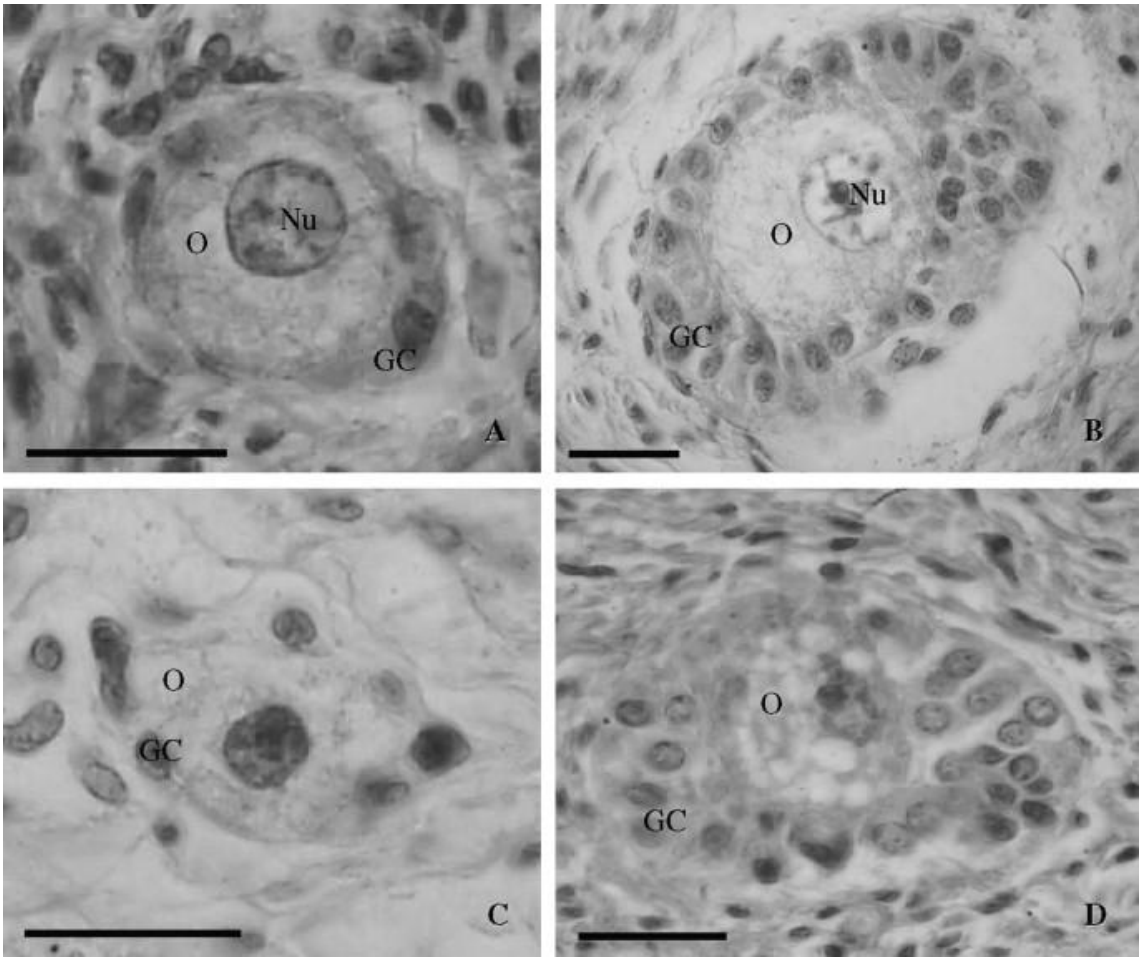

Fig. 5. Histological sections of preantral follicles. (A) Morphologically normal early preantral follicle cryopreserved with Me2SO. (B) Morphologically normal growing follicle cryopreserved with EG. (C) Degenerated early preantral follicle cryopreserved with PROH after short-term tissue incubation, note the disorganization of granulosa cells. (D) Degenerated growing follicle cryopreserved with PROH, note the oocyte with a pycnotic nucleus and vacuolated cytoplasm. O: oocyte, GC: granulosa cells, Nu: nucleus. Barr = 20 µm.

The ultrastructural analysis showed that follicles from control group (Fig. 6A) presented oocytes with a large central nucleus well-delimited by the nuclear envelope. Organelles were uniformly distributed throughout the cytoplasm. Round mitochondria were the most evident organelle. A small number of elongated mitochondria were also observed in some cases. A few cisternae of smooth endoplasmic reticulum, lipid droplets and vesicles were also seen evenly distributed throughout a homogeneous cytoplasm.

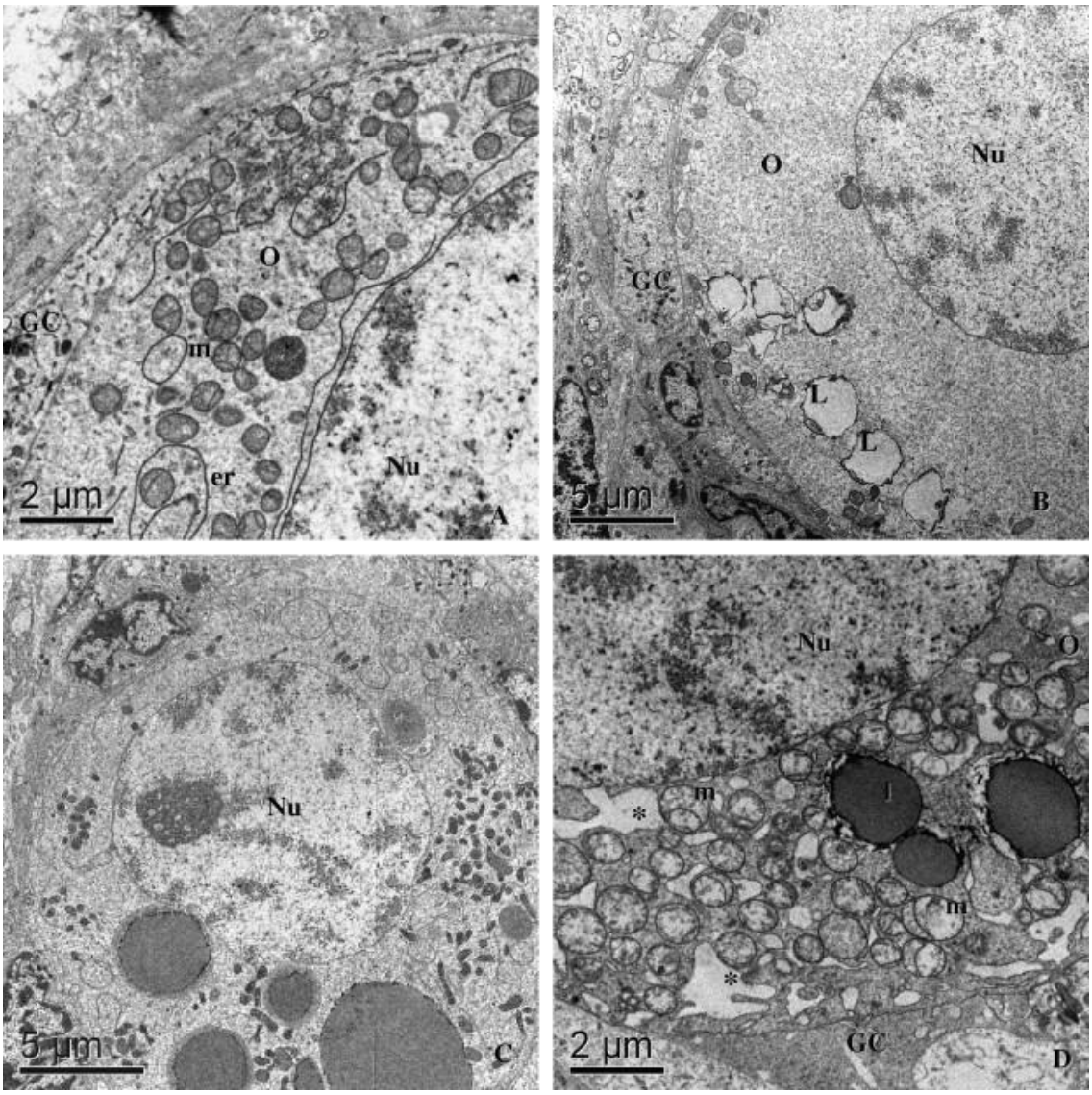

Fig. 6. Electron micrographs of follicles from control (A) and cryopreserved with EG (B) and Me2SO before (C) and after STTI (D). Note the normal ultrastructure (A and C), with round mitochondria and endoplasmic reticulum cisternae. The main alterations observed in cryopreserved follicles were swollen mitochondria (D). Oocyte cytoplasm in cryopreserved follicles also presented a granulated appearance (B) and some empty spaces (D). O: oocyte, GC: granulosa cells, Nu: nucleus, l: lipid droplets, m: mitochondria, er: endoplasmic reticulum cisternae, *: empty spaces.

Follicles from the STTI control showed an ultrastructure similar to the control (Fig. 6A), although some mitochondria lost their cristae and presented a granulated matrix. Moreover, some follicles showed empty spaces in the ooplasm.

In general, the ultrastructure of follicles cryopreserved with Me2SO or EG, both before and after short-term tissue incubation, was similar. Although their ultrastructure was not very different (Fig. 6C) from control follicles, some alterations could be observed. Oocyte cytoplasm in cryopreserved follicles sometimes presented a granulated appearance (Fig. 6B) and some empty spaces (Fig. 6D). Swollen mitochondria (Fig. 6D) could also be seen. In all follicles analyzed, granulosa cells presented a normal appearance.

Discussion

This study describes, for the first time, the effect of different cryoprotectants on cryopreservation of preantral follicles in swine ovarian tissue. Similar comparative studies were performed on ovarian tissue from humans [26], ovines [3], [4] and [24], caprines [22] and [25], bovines [5] and [16] and felines [14].

In the present study, swine ovarian tissue was better preserved in Me2SO or EG than in PROH or GLY. Me2SO and EG have been reported as the best cryoprotective agents for cryopreservation of ovarian tissue in many species (bovine [5] and [16], caprine [22] and [25] ovine [3], [4] and [24], humans [26] and feline [14]). When compared with each other, some authors found that EG was better than Me2SO for cryopreserving ovine, bovine and caprine ovarian tissue [3], [4], [5] and [25]. However, some other studies with bovines [16] and ovines [24] showed that Me2SO presented better results than EG.

When penetration rates of GLY, EG, Me2SO and PROH were compared, results indicated EG and Me2SO as the best ovarian tissue cryoprotectants, as they have lower molecular weight, which permits a faster penetration compared with PROH and GLY [19]. According to the same author, the extent of follicular survival is at least in part determined by the speed of cryoprotectant permeation. Because most cells are relatively impermeable to GLY, its use might lead to severe osmotic effects and consequently it is not indicated for several different systems, including ovarian tissue [1].

A significant effect of the post-thaw short-term tissue incubation was only observed for early preantral follicles cryopreserved with Me2SO, where an increase in the percentage of MNF was observed after the 2 h incubation. This suggests that some follicles classified as degenerated might have recovered their normal morphology after the incubation. Paynter et al. [21] stated that a short term period (1–4 h) of post-thaw culture allows follicular cells to reestablish metabolic activity, normal cell volume control, and cell–cell contacts. These authors found in their study that the process of rewarming cryopreserved tissue in culture medium for 1 h enhanced follicle recovery.

Our study also demonstrated that although ovarian tissue cryopreserved with EG and Me2SO presented high percentage of morphologically normal follicles using histological analysis, these follicles sometimes showed minor alterations when evaluated by transmission electron microscopy. Previous studies on frozen-thawed ovarian tissue [16] and [28] reported that histology results are not always confirmed by ultrastructural analysis.

In the present study, discreet changes in the ultrastructure, such as swollen mitochondria and some void areas, were seen in pig preantral follicles cryopreserved with Me2SO and EG. Such areas in the oocyte cytoplasm may represent endoplasmic reticulum swelling [29] and [31]. Swollen endoplasmic reticulum and mitochondria were already described in oocytes [28] and [32] luteal cells [6] and [9] and other cell types [8] and [11]. The swelling of mitochondria and endoplasmic reticulum are described as a consequence of changes in ionic balance caused by altered plasma membrane permeability [6]. In the present experiment, these alterations may be due to osmotic effects related to the cryoprotective agents or the cryopreservation process. Although swollen mitochondria could affect cellular metabolism [11] and [23], this change was showed to be reversible in a short time period [12] and [13]. Discreet changes in the ultrastructure of preantral follicles were also seen in sheep ovarian tissue cryopreserved with EG [25]. According to Silva et al. [28], swelling of mitochondria and endoplasmic reticulum with increased volume are very early signs of oocyte degeneration. Signs of advanced degeneration were not observed in the present study.

Although in this work some ultrastructural alterations were observed in the oocytes of cryopreserved follicles, granulosa cells always showed a normal appearance. It is already known that, in preantral follicles, the oocyte is more sensitive to degeneration than granulosa cells [2].

Although morphological evaluation is very useful to access the extent of damage in cells submitted to cryopreservation protocols [17], it is not always correlated to the viability or developmental competence of the follicles [32]. Therefore, a more comprehensive evaluation of cryodamage should be assessed using other methods [3], such as viability tests or long term culture to achieve growth and development.

In conclusion, the present study showed that preantral follicles in swine ovarian tissue were better cryopreserved using Me2SO or EG than PROH or GLY. Although more studies must be carried out to determine an optimal method to cryopreserve pig preantral follicles in ovarian tissue, the present work represents an advance in this field. Since the cryopreservation of pig oocytes from antral follicles show very poor results, the cryopreservation of preantral follicles represents a good alternative in this species and must be better studied.

Acknowledgments

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