Ultrastructural features of agouti (Dasyprocta aguti) preantral follicles cryopreserved using dimethyl sulfoxide, ethylene glycol and propanediol

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Abstract

The objective was to develop an efficient protocol for cryopreservation of agouti (*Dasyprocta aguti*) ovarian tissue. Agouti ovarian fragments were placed, for 10 min, in a solution containing MEM and fetal bovine serum plus 1.5 M dimethyl sulfoxide (DMSO), ethylene glycol (EG) or propanediol (PROH); some of those fragments were subsequently cryopreserved in a programmable freezer. After exposure and/or thawing, all samples were fixed in Carnoy prior to histological analysis. To evaluate ultrastructure, follicles from the control and all cryopreserved treatments were fixed in Karnovsky and processed for transmission electron microscopy. After exposure and freezing, there was a significant decrease in the percentage of morphologically normal preantral follicles in all treatments when compared to the control (92.67 ± 2.79, mean ± SD). However, there were no significant difference when the exposure and freezing procedures were compared using the same cryoprotectant. Moreover, there was no significant difference among cryoprotectants at the time of exposure (DMSO: 64.7 ± 3.8; EG: 70.7 ± 11.2, PROH: 63.3 ± 8.5) or after freezing (DMSO: 60.6 ± 3.6, EG: 64.0 ± 11.9; PROH: 62.0 ± 6.9). However, only follicles frozen with PROH had normal ultrastructure. In conclusion, preantral follicles enclosed in agouti ovarian tissue were successfully cryopreserved using 1.5 M PROH, with satisfactory maintenance of follicle morphology and ultrastructure.

Keywords: Preantral follicles; Freezing; Agouti; Ultrastructure; Follicle morphology

1. Introduction

Cryopreservation of ovarian tissue has been widely studied to preserve the large contingent of female gametes present in preantral follicles (PAF), which are considered the ovarian follicle reserve. Various cryoprotectant agents (CPAs), e.g., ethylene glycol, dimethyl sulfoxide and propanediol, have been successfully used for PAF cryopreservation in various

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animals, including sheep [1], goats [2,3], primates [4], rodents [5], and humans [6]. Satisfactory results have been reported, especially when a 1.5 M concentration was used in slow-freezing protocols. An important application of ovarian tissue cryopreservation is preservation of female gametes of endangered species, which can be stored in germplasm banks and, thereafter, be cultured in vitro or transplanted to support ART that require mature oocytes.

Nevertheless, due to the low availability of endangered species for use in scientific research, it is necessary to use experimental models, usually domestic animals or animals that are not under the threat of extinction, that are phylogenetically close to the species of interest [7]. Hamelett and Rasweiler [8] highlighted the importance of finding new animal species with the potential to be used as experimental models, to support the development of vital research to endangered animals and even humans. In this context, we highlight agouti (Dasyprocta aguti) because it has characteristics that are important for an ideal experimental model for wild rodents threatened with extinction, including small size, low maintenance costs, and a short gestation [9]. Furthermore, agoutis also represent an alternative protein source with the potential for economic exploitation [10]. Therefore, obtaining information regarding reproduction of this species will also provide information for their rational production and consequent preservation [11]. In this regard, several studies have been performed involving the reproductive characteristics of agoutis, such as placentation [10], morphology of the ovaries [12] and oviducts [13], semen collection, and semen cryopreservation [14,15].

The development of a cryopreservation protocol of agouti ovarian tissue might be of great importance for this technique to be applied to the preservation of this species and also to other endangered rodents. However, there are apparently no available studies on the cryopreservation of agouti ovarian tissue. Thus, the objective of this study was to assess the feasibility of the cryopreservation of preantral follicles enclosed in the ovarian tissue of these animals by testing various CPAs, including dimethyl sulfoxide (DMSO), propanediol (PROH) and ethylene glycol (EG), and assessing follicle morphology and ultrastructure.

2. Materials and methods

2.1. Animals

The Animal Use Ethics Committee of the State University of Ceará approved this study. The agoutis (Dasyprocta aguti) used during the experiment belonged to the Centre of Multiplication of Wild Animals from Rural Federal University of Semi-Arid, located in the northeast of Brazil

(Mossoró, RN, Brazil; 5°10=S, 37°10=W). The climate there is typically semi-arid with a mean annual temperature of 27 °C. The animals were fed seed and fruits, with ad libitum access to water.

2.2. Source and preparation of ovaries

Pairs of ovaries (n = 5) were collected immediately after euthanasia, rinsed once with 70% ethanol for 10 s and twice in HEPES-buffered Minimum Essential Medium (H-MEM, osmolarity 280 mOsmol/L, pH 7.2; Sigma, St. Louis, MO, USA) and transported to the laboratory in 50-mL tubes (Corning Glass Works, Corning, NY, USA) containing 15 mL of H-MEM at 4 °C within 1 h after collection.

2.3. Exposure and freeze thaw procedures

In the laboratory, ovaries were divided into four equal portions (approximately 4 4 1 mm); two pieces from each ovarian pair were randomly selected to be the control and were immediately fixed for histological and ultrastructural analysis. The remaining pieces were individually placed in 2-mL maxi-straws (MINITUB do Brasil Ltda., Porto Alegre, RS, Brazil) containing 1.8 mL of HMEM (HMEM 10% fetal bovine serum; Laborclin, Pinhais, PR, Brazil) to which either 1.5 M dimethyl sulfoxide (DMSO), ethylene glycol (EG) or propanediol (PROH) was added. Exposure of ovarian fragments to the described solutions was performed at 20 °C for 10 min. After exposure, three ovarian fragments were subjected to cryoprotectant removal from the tissue at room temperature by three washes (5 min each) in MEM with 10% FBS and decreasing sucrose concentrations (0.5, 0.25, and 0 M). The remaining fragments were cryopreserved using a programmable freezer (Freeze Control, CryoLogic Pty Ltd., Waverley, Australia) as previously described by Faustino et al [16]. Briefly, maxi-straws were cooled at a rate of 2 °C/min from 20 to 7 °C, and then ice-induction (seeding) was manually performed by touching the maxistraws with forceps that had been pre-cooled in liquid nitrogen. After seeding, the maxi-straws were held at this temperature (7 °C) for 10 min, cooled at a rate of 0.3 °C/min to 40 °C, and then finally cooled at a rate of 10 °C/min to 70 °C, after which the maxi-straws were plunged immediately into liquid nitrogen (196 °C) and stored for approximately 1 wk. When required, the maxi-straws were thawed in air for 1 min at room temperature (25 °C) and then immersed in a water bath at 37 °C until the cryoprotectant solution had completely melted. The cryoprotectant was then removed, as described above for the exposed ovarian tissue. After these procedures, all ovarian fragments L.S. Wanderley et al. /

Theriogenology 77 (2012) 260–267 261 were fixed for histological and ultrastructural analysis (Fig. 1). Each treatment was repeated five times.

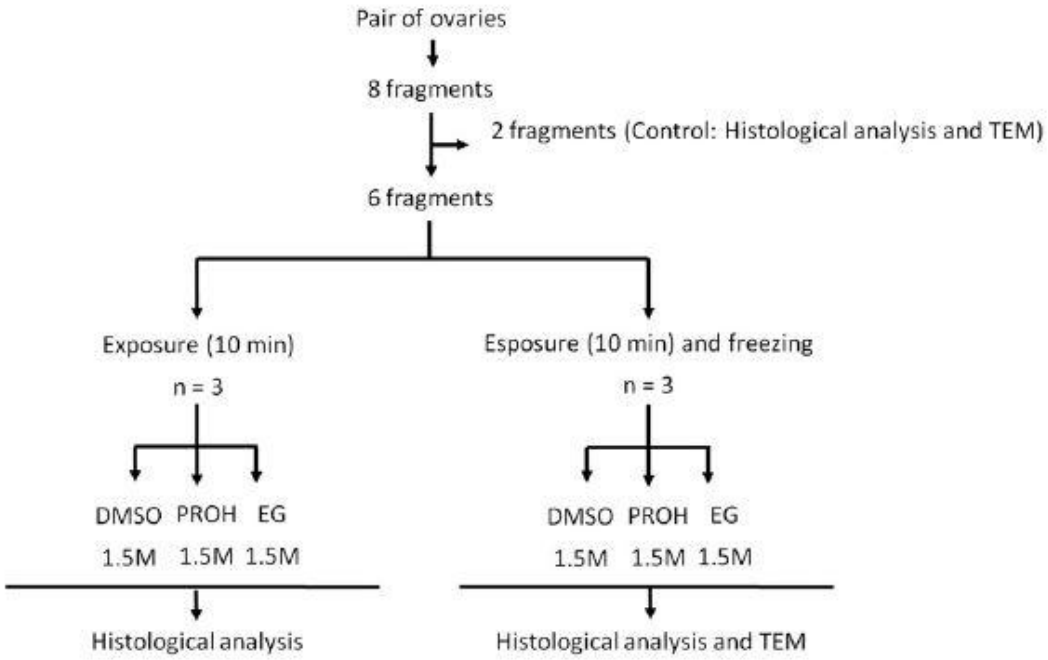


Fig. 1. Experimental design for exposure and freezing of agouti ovarian tissue. TEM, transmission electron microscopy.

2.4. Histological analysis

To assess morphology of cryopreserved agouti preantral follicles, fragments of ovarian tissue were processed according to Faustino et al [16], with some modifications. Briefly, samples were fixed in Carnoy for 4 h, dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax, and serially sectioned (7 µm). Every fifth section was mounted on a glass slide, stained with hematoxylin and eosin (HE) and evaluated by light microscopy (Nikon, Tokyo, Japan) at 400x.

Preantral follicles were defined as an oocyte surrounded either by one layer of flattened or cuboidal granulosa cells, or several layers of cuboidal granulosa cells with no antrum. Only preantral follicles with visible nuclei were counted. Follicle morphology was evaluated based on the integrity of the oocyte, granulosa cells, and basement membrane. Preantral follicles were classified and counted as: (i) morphologically normal (MNPF) if they contained an oocyte with regular shape and uniform cytoplasm and organized layers of granulosa cells; or as (ii)

degenerated if they exhibited disorganization of granulosa cells, a shrunken oocyte, oocyte cytoplasm vacuolization, or low granulosa cell density.

2.5. Ultrastructural analysis

To compare the ultrastructure of preantral follicles from the control, as well as from treatment groups with higher percentages of MNPF in the light microscopy evaluation, evaluation with transmission electron microscopy (TEM) was performed as described [17], with some modifications. Portions (maximum size, 1 mm3) were cut from each fragment of ovarian tissue and fixed in a modified Karnovsky solution (2% paraformaldehyde and 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer at a pH of 7.2) for 3 h at room temperature (RT, approximately 25 °C). After three washes in sodium cacodylate buffer, specimens were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h at RT. Samples were then dehydrated through a gradient of acetone solutions and, thereafter, embedded in Spurr's epoxy resin. Afterwards, semithin sections (5 m) were cut, stained with toluidine blue, and analyzed by light microscopy at a 400x magnification. Ultra-thin sections (60 – 80 nm) were obtained from preantral follicles that were classified as morphologically normal in semi-thin sections, according to the criteria adopted for histology. Subsequently, ultra-thin sections were contrasted with uranyl acetate and lead citrate, and examined under a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan) operating at 80 kV.

2.6. Statistical analysis

Analysis of variance was done using the GLM procedure of SAS (1999), according to a 3×2 factorial arrangement of treatments with the three cryoprotectants (DMSO, EG, and PROH) and two procedures (exposure only or exposure followed by cryopreservation) as the main effects. Differences among treatments were determined by a Student-Newman-Keuls procedure. The results were presented as the mean ± SD and the differences were considered significant when $P < 0.05$.

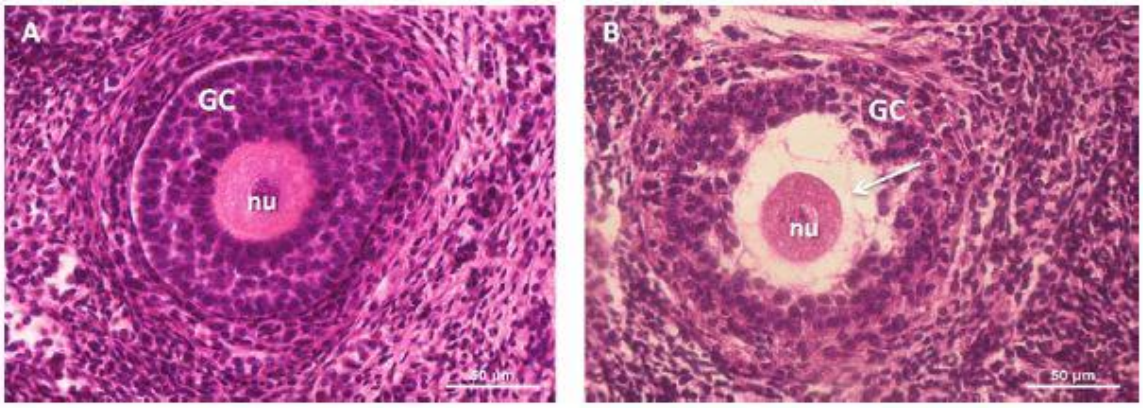


Fig. 2. Histological sections of agouti preantral follicles. (A) Morphologically normal follicle (control); (B) Degenerated follicle, cryopreserved with EG. Note retraction of the oocyte cytoplasm (arrow) and disorganized granulosa cells. Nu, oocyte nucleus; gc, granulosa cells.

3. Results

3.1. Histological analysis

A total of 1,050 preantral follicles were analyzed (150 follicles per treatment in five replicates). In general, normal (Fig. 2A) and degenerated (Fig. 2B) follicles were present in the control and all treatments. The percentages of MNPF found in the control and the treatments that were only exposed and/or cryopreserved in the presence of 1.5 M of DMSO, EG, or PROH are shown (Fig. 3). In frozen-thawed tissues, there was a significant reduction in the percentage of morphologically normal follicles in all treatments compared to the control (92.7% ± 2.8). However, there were no significant differences when the procedures of exposure and freezing were compared to each other. Similarly, there were no significant differences among cryoprotectants after exposure only (DMSO: 64.7 ± 3.8%; EG: 70.7% ± 11.2, and PROH: 63.3% ± 8.5) or after exposure followed by freezing (DMSO: 60.6% ±3.6, EG: 64.0% ± 11.0; and PROH: 62.0% ± 6.9).

3.2 Ultrastructural analysis

Based on TEM, normal preantral follicles had an oocyte with homogeneous cytoplasm that was full of mitochondria with continuous membranes and peripheral cristae. Some elongated mitochondria with parallel cristae were also detected, whereas small Golgi complexes were rarely observed. Smooth and rough endoplasmic reticulum were present, alone or in association with the mitochondria. The oocyte nucleus was large and well delimited by the nuclear envelope and

contained decondensed chromatin in some areas and aggregates of chromatin, and the nucleolus was frequently identified. Granulosa cells were small and had a high nucleus-cytoplasm ratio. Irregularly shaped nuclei of these cells contained decondensed chromatin on the inside and condensed chromatin in the periphery. The cytoplasm had numerous mitochondria and well developed smooth and rough endoplasmic reticulum. Membranes of the oocyte and the surrounding granulosa cells were closely juxtaposed. A distinct, continuous basement membrane surrounded the follicles and was strongly adhered to the stroma. A normal (Fig. 4A) and a degenerated (Fig. 2B) follicle are shown in Fig. 4. The normal pattern of ultrastructure was observed in the follicles of the control (Fig. 5A) and those cryopreserved with PROH (Fig. 5B), which proved to be the most effective cryoprotectant. However, changes were detected in the follicles that were cryopreserved with DMSO and EG and that had been considered normal in semithin sections when visualized with light microscopy. In follicles cryopreserved in the presence of DMSO, numerous vacuoles were seen in the ooplasm, in addition to a high degree of vacuolization in granulosa cells and the loss of the cytoplasmic content of these cells, leading to the formation of large void spaces in some cases (Fig. 5C). In follicles cryopreserved with EG, the ooplasm had reduced electron density and a small number of organelles that were heterogeneously distributed into small groups (Fig. 5D).

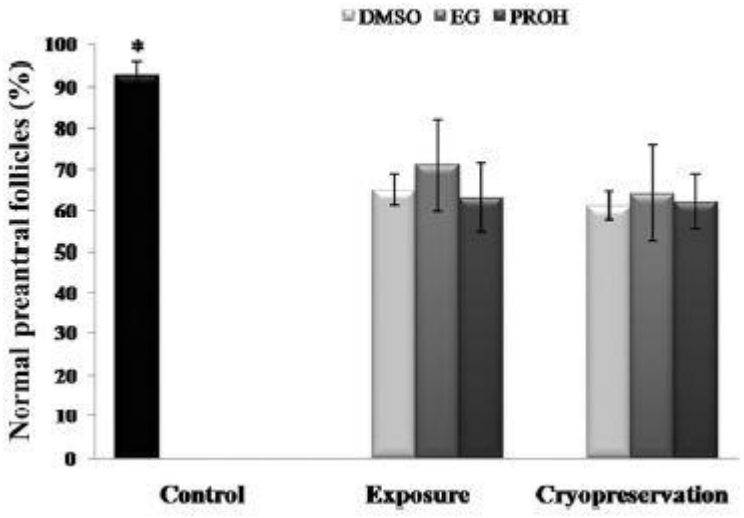


Fig. 3. Percentage of morphologically normal follicles in the control and after exposure to cryoprotectants (DMSO, EG or PROH) for 10 min, with or without subsequent cryopreservation. *Differs from treatments ($P < 0.05$).

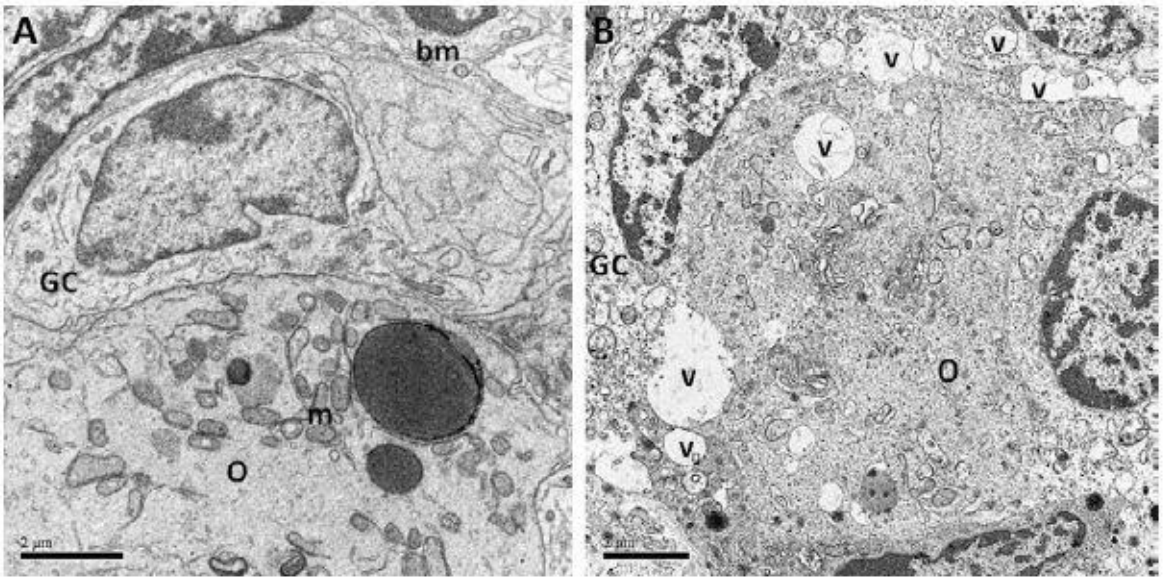


Fig. 4. Electron micrographs of a normal preantral agouti ovarian follicle from a control group (A, 12000x), and a frozen-thawed follicle with 1.5 M EG follicle (B, 10000x), displaying ultrastructural alterations. In Fig. 4B, note the intense ooplasm vacuolization. O, oocyte; GC, granulose cells; m, mitochondria; bm, basement membrane; v, vacuole.

4. Discussion

The current study demonstrated, apparently for the first time, the cryopreservation of agouti (Dasyprocta aguti) ovarian tissue, using three cryoprotectants. In an attempt to choose the most suitable cryoprotectant agent to promote the cryoprotection of the agouti ovarian tissue and eliminate freezing-induced damage, PROH, DMSO, and EG were used, which have been widely used with success at concentrations of 1.5 M [18]. In addition, this was apparently also the first study to report ultrastructural features of fresh and cryopreserved agouti preantral follicles.

Exposure of ovarian tissue fragments to 1.5 M of the three tested cryoprotectants (DMSO, EG, or PROH) significantly reduced percentages of preantral follicles with normal morphology compared to the control or fresh fragments. Similar changes were reported following exposure of ovarian tissue of sheep [19] and goats [2,3] to 1.5 M concentrations of the same cryoprotectants. Despite protecting cells and tissues against cryoinjuries [20], the CPAs may cause damage by their toxic action [21], which can vary with concentration, time, and exposure temperature [22].

In the present study, the percentage of morphologically normal preantral follicles in the fragments that were only exposed to a CPA was similar to that observed after freezing and thawing. Therefore, we inferred that the reduction in the number of PAFs occurred mainly as a

result of the exposure of the normal tissue to the cryopreservation solution, with agouti ovarian tissue being quite resistant to extreme reductions in temperature.

Follicle morphology, analyzed by classical histology, was not significantly different among the three cryoprotectants, tested in terms of the percentage of normal follicles after cryopreservation. However, with TEM, changes in the ultrastructure of cryopreserved follicles were apparent. Similar changes were reported after cryopreservation of porcine ovarian tissue with 1.5 M EG and DMSO [23]. It was noteoworthy that TEM was considered a good technique for analysis of cellular organelles and ultrastructural changes in cryopreserved ovarian tissue [24] and thus, was a better evaluation method than classical histology.

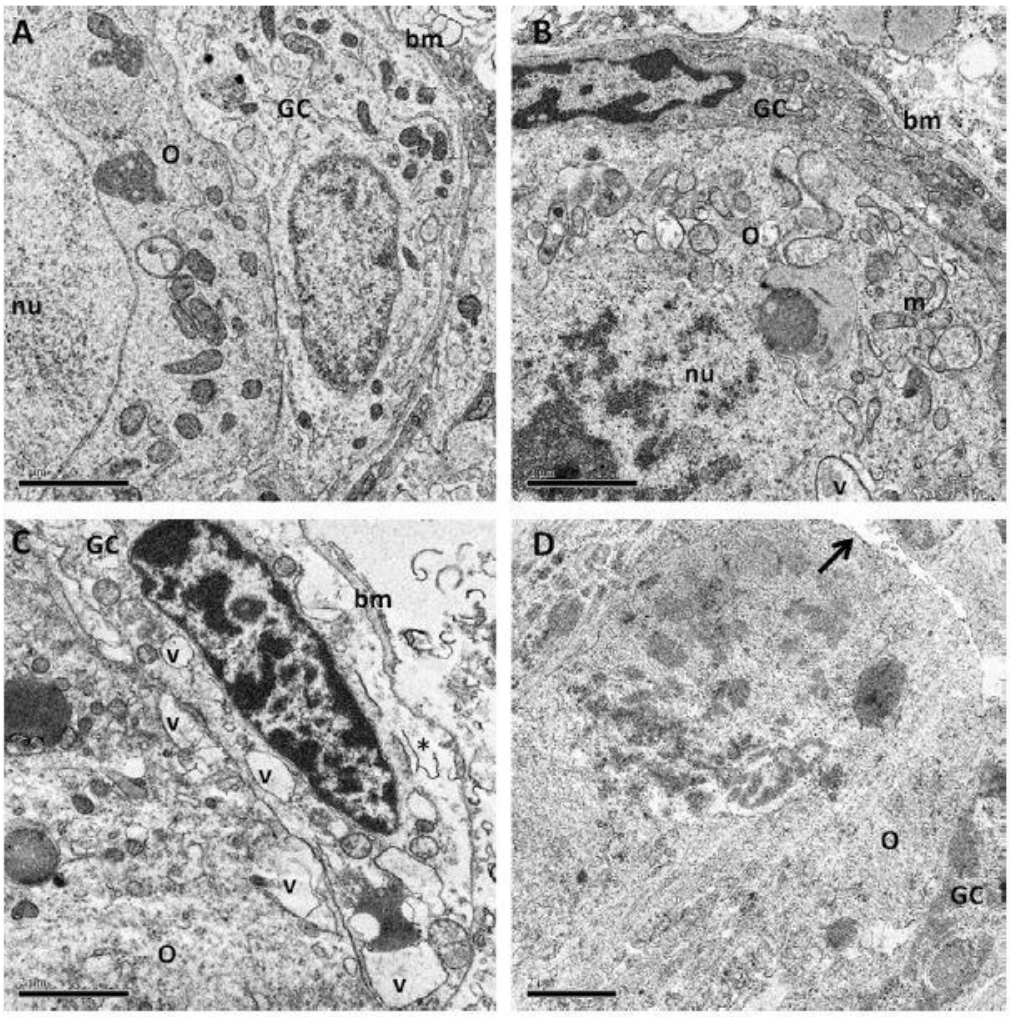
Follicle ultrastructure was better preserved in the presence of PROH, which is considered a low-toxicity cryoprotectant that can protect cells against freezing- induced dehydration [25] and thus is one of the CPAs that is most often used for cryopreservation of human oocytes [26]. In previous studies, 1.5 M of this cryoprotectant yielded satisfactory results for cryopreservation of preantral follicles enclosed in the ovarian tissue of rabbits [27] and sheep [28]. In a recent study by our team, ovarian tissue of goats was successfully cryopreserved in the presence of 1.0 M PROH (unpublished data). In contrast, Oskam et al [29], reported that PROH caused deleterious effects to the stroma and cryopreserved PAFs of ovine ovarian tissue.

In the present study, ovarian tissue cryopreserved with DMSO had a greater degree of vacuolization in both the ooplasm and granulosa cells, which coalesced to form voids. Unlike results previously reported in our studies in pigs [23], cattle [30], and sheep [19], DMSO was able to maintain the ultrastructure of preantral follicles enclosed in ovarian tissue after cryopreservation. In follicles included in ovarian tissue that were cryopreserved in the presence of EG, greater damage was observed in this study, manifested as reduced electron density and fewer organelles in the ooplasm. These findings differed from those in goats [2] and cattle [31], in which the cryoprotectant agent was highly efficient in maintaining follicle ultrastructure.

We speculated that apparent differences between the results obtained in this study and those previously reported were due to differences between protocols and the efficiency of the cryoprotectants. In that regard, the latter was closely related to the organization of ovarian tissue and associated with species-specific characteristics [32], not allowing for extrapolation of protocols for species that are phylogenetically distant.

In conclusion, preantral follicles enclosed in agouti ovarian tissue were successfully cryopreserved using 1.5 M PROH; the rate of MNPF was satisfactory and ultrastructure was best

preserved with this CPA. This protocol should be of great importance both for the preservation of the species itself and for other endangered wild rodents whose ovaries have similar characteristics.



Acknowledgments

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