Short-term preservation of canine preantral follicles: Effects of temperature, medium and time

Cláudio Afonso Pinho Lopes
Regiane Rodrigues dos Santo
Juliana Jales de Hollanda Celestino
Mônica Aline Parente Melo
Roberta Nogueira Chaves
Claudio Cabral Campello
José Roberto Viana Silva
Sônia Nair Báo
Katarina Jewgenow
José Ricardo de Figueiredo

Abstract

The use of the large pool of preantral follicles is a promising alternative to provide high numbers of fertilizable oocytes to reproductive biotechnology. This issue is particularly important to canids, since current rates of success of in vitro techniques using oocytes are very limited, and many species within this family are threatened by extinction. The aim of this study was to evaluate effects of temperature, medium and time on morphology and viability of canine preantral follicles during short-term preservation. Canine ovaries were cut into fragments which were incubated in 0.9% NaCl solution or in minimum essential medium (MEM) at 4, 20 or 38 °C for 2, 6, 12 or 24 h. Afterwards, preantral follicles were analyzed by histology, transmission electron microscopy and viability testing using trypan blue, calcein-AM and ethidium homodimer-1. Percentages of morphological normal and viable follicles were maintained similar to control (time 0 h) after incubation in 0.9% NaCl at 4 or 20 °C for up to 6 h and at 38 °C for 2 h. Using MEM, such preservation was possible for 12 h at 4 or 20 °C, and for 6 h at 38 °C. These results indicate that preservation of canine preantral follicles might be better accomplished through hypothermic (4 or 20 °C) storage in MEM, which ensures maintenance of morphology and viability for up to 12 h.

Keywords: Preservation; Hypothermia; Morphology; Viability; Preantral follicle; Canine

1. Introduction

Reproductive biotechnology has a great potential to contribute for the conservation of endangered wildlife species. However, a limiting factor for the development and efficiency of reproductive techniques is the lack of abundant numbers of fertilizable oocytes. This problem could be addressed by using the large source of oocytes enclosed in preantral follicles (Telfer, 2001), which exist in ovaries in numbers of thousands to millions depending on the species. In vitro culture systems capable to promote growth and maturation of these follicles have been

developed for several species, and birth of healthy offspring after embryo transfer of in vitro fertilized oocytes derived from primordial follicles has, so far, been achieved in mice (Eppig and Schroeder, 1989).

Due to the fact that time from ovary collection to the outset of in vitro culture can be very long, especially when the donor animal is far from the reproductive laboratory, as is the case with free-ranging individuals in the wild, preservation of viability is a critical issue since cells are under ischemia. In this context, studies have assessed short-term preservation of preantral follicles combining different temperatures and media in several species such as caprine (Silva et al., 2000, Silva et al., 2001, Carvalho et al., 2001 and Ferreira et al., 2001), ovine (Andrade et al., 2001, Andrade et al., 2002a, Andrade et al., 2002b and Matos et al., 2004), bovine (Lucci et al., 2004) and swine (Lucci et al., 2007).

Conversely, to our knowledge, there is not to date a study on short-term preservation of canine preantral follicles. Few works have addressed the storage of canine ovaries, but only effects on in vitro maturation (IVM) of oocytes from antral follicles have been evaluated and findings are controversial. Taş et al. (2006) demonstrated that bitch oocytes maintained at 4 °C in physiologic saline had higher maturation rates than those kept at 35–38 °C. In contrast, Lee et al. (2006) observed that viability of canine antral oocytes after in vitro maturation was significantly decreased when ovaries were stored at 4 °C in relation to 38 °C.

Thus, methods for the preservation of canine oocytes are still to be established, and more studies are needed to gather consistent data. Once established, in vitro embryo production in dogs based upon the vast pool of preantral follicles could be adapted to endangered wild canids, which would contribute enormously to the multiplication of individuals of such species. Nine canid species are under risk of extinction, which has already occurred to the Falklands Wolf, Dusicyon australis (IUCN, 2007).

In this context, the aim of this study was to assess the preservation of canine preantral follicles maintained in physiologic saline solution or in minimum essential medium (MEM) at different temperatures and times of storage. To this end, evaluation of morphology through histological and ultrastructural analyses, as well as viability assays based on trypan blue dye exclusion and the fluorescent probes calcein-AM and ethidium homodimer were performed.

2. Materials and methods

2.1. Source and preparation of canine ovaries

Pairs of ovaries (n = 15) were aseptically collected during ovariectomy of healthy mixed-breed bitches (Canis familiaris) whose reproductive history and age were unknown, but it was estimated that most were 12–24 months old and believed nulliparous. After removal from the bursa ovarica, ovaries were rinsed once with 70% ethanol for 10 s, twice in sterile phosphate-buffered saline (PBS) and eventual corpora lutea were excised using a scalpel.

2.2. Experimental design

2.2.1. Experiment I: morphological evaluation of stored canine preantral follicles

Immediately after collection and preparation, pairs of ovaries (n = 5) were divided each into 25 fragments, from which one was randomly selected as control (time 0 h) and fixed for histology and ultrastructural analysis. The remaining fragments were placed into 15 ml tubes (Corning Glass Works, Corning, NY, USA) containing 2 ml of sterile saline solution (0.9% NaCl solution, osmolarity 300 mOsmol/l, pH 7.2) or HEPES-buffered MEM (osmolarity 280 mOsmol/l, pH 7.2; Sigma, St. Louis, MO, USA) at 4, 20 or 38 °C and stored for 2, 6, 12, or 24 h, and subsequently fixed for morphological analysis as the non-stored control sample (Fig. 1). Thermoflasks filled with water were used for maintaining temperatures, which were monitored throughout the experiment. Each treatment was repeated five times.

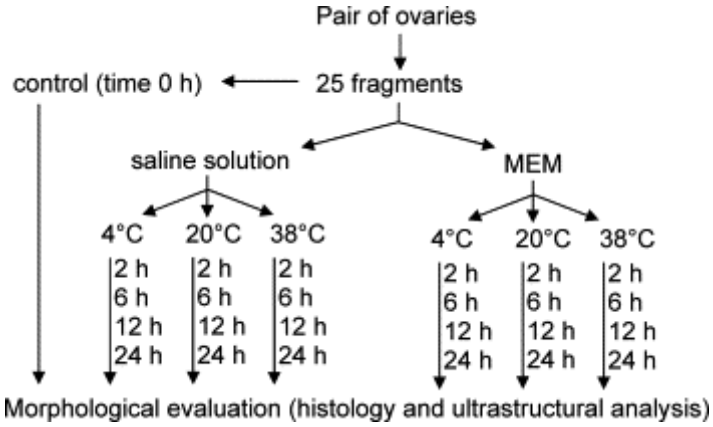

Fig. 1. Design of experiment I: pairs of canine ovaries were divided into 25 fragments, being one selected as non-stored control (time 0 h), whilst the others were incubated in saline solution or in minimum essential medium (MEM) at 4, 20 or 38 °C for 2, 6, 12, or 24 h. Afterwards, fragments were submitted to morphological analysis.

2.2.2. Experiment II: assessment of viability of stored canine preantral follicles

In this study, effects of storage on canine preantral follicles were further analyzed through assessment of viability. Five pairs of canine ovaries were cut each into 25 fragments, and one was randomly selected and immediately submitted to follicle isolation followed by trypan blue dye exclusion test as described later. The other fragments were maintained in saline solution or in MEM at 4, 20 or 38 °C for 2, 6, 12 or 24 h, and afterwards evaluated similarly as for the control.

Based on results of experiment I and trypan blue test, a second viability trial was performed. Pairs of ovaries (n = 5) were cut into three portions, from which one was immediately processed for follicle isolation and testing using trypan blue as well as calcein-AM and ethidium homodimer, which was employed to provide an additional and more precise method of viability assessment. The remaining fractions were stored in MEM at 4 °C for 12 and 24 h, and afterwards analyzed similarly as for the control.

2.3. Histological analysis

In order to assess the morphology of canine preantral follicles submitted to the different treatments, fragments of ovarian tissue were fixed in Carnoy for 12 h, dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax and serially sectioned at 7 μm. Every fifth section was mounted on glass slides, stained with periodic acid Schiff (PAS)-hematoxylin and evaluated by light microscopy at a 400× magnification (Zeiss, Germany). 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. Follicular 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, when containing an oocyte with regular shape and uniform cytoplasm, and organized layers of granulosa cells, or (ii) degenerated, when the oocyte exhibited pycnotic nucleus and/or ooplasma shrinkage, and occasionally granulosa cell layers became disorganized, detached from the basement membrane and/or included enlarged cells. To avoid evaluating and counting a follicle more than once, preantral follicles were analyzed only in the sections where oocyte nucleus was observed.

2.4. Ultrastructure analysis

In order to better examine follicular morphology, transmission electron microscopy (TEM) was performed to analyze ultrastructure of preantral follicles from the control, as well as from treatments that did not differ statistically from control in histology. A portion with a maximum dimension of 1 mm3 was cut from each fragment of ovarian tissue and fixed in Karnovsky solution (2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 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. The samples were then dehydrated through a gradient of acetone solutions and thereafter embedded in Spurr's epoxy resin. Afterwards, semi-thin sections (3 μm) were cut, stained with toluidine blue and analyzed by light microscopy at a 400× magnification. Ultra-thin sections (60–70 nm) were obtained from preantral follicles classified as morphologically normal in semi-thin sections, according to the criteria adopted in histology. Subsequently, ultra-thin sections were contrasted with uranyl acetate and lead citrate, and examined under a Jeol 1011 (Jeol, Tokyo) transmission electron microscope operating at 80 kV.

2.5. Assessment of preantral follicles viability

Canine preantral follicles were isolated from control and stored ovarian fragments using the mechanical method described by Figueiredo et al. (1993). Briefly, using a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to a sectioning interval of 87.5 μm, samples were cut into small pieces, which were placed in MEM and suspended 40 times using a large Pasteur pipette (diameter of about 1600 μm) and subsequently 40 times with a smaller Pasteur pipette (diameter of approximately 600 μm) to dissociate preantral follicles from stroma. The obtained material was passed through 500- and 100-μm nylon mesh filters, resulting in a suspension containing preantral follicles smaller than 100 μm in diameter. This procedure was carried out within 10 min at RT.

Thereafter, viability of preantral follicles was assessed through trypan blue dye exclusion test (Jewgenow et al., 1998). Briefly, 10 μl of 0.4% trypan blue (Sigma, Deisenhofen, Germany) were added to 90 μl of the suspension of isolated preantral follicles, which were examined using an inverted microscope after incubation for 5 min at RT. Follicles were

classified as viable if the oocyte and <10% granulosa cells remained unstained, or as non-viable if uptake of the dye by the oocyte and/or ≥10% granulosa cells occurred.

Preantral follicles were also analyzed using a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells by calcein-AM and ethidium homodimer-1, respectively. Whilst the first probe detected intracellular esterase activity of viable cells, the later labeled nucleic acids of non-viable cells with plasma membrane disruption. Since it is difficult to distinguish the outline of live cells labeled with calcein-AM within follicle structure, determination of the percentage of viable granulosa cells was achieved through using Hoechst 33343 to detect all nuclei, enabling counting of the total number of cells. The test was performed by adding 4 µM calcein-AM, 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) and 10 µM Hoechst 33342 (Sigma, Deisenhofen, Germany) to the suspension of isolated follicles, followed by incubation at 37 °C for 10 min. After being labeled, follicles were washed three times by centrifugation at 100 × g for 5 min and resuspension in MEM, mounted on a glass microscope slide in 5 µl antifading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching, and finally examined using an a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescent signals of Hoechst, calcein-AM, and ethidium homodimer were collected at 350, 488, and 568 nm, respectively. Oocytes and granulosa cells were considered live if the cytoplasm was stained positively with calcein-AM (green) and chromatin was not labeled with ethidium homodimer (red). Preantral follicles were classified as viable when the oocyte and <10% of granulosa cells were live, and as non-viable, when the oocyte and/or ≥10% granulosa cells were dead.

2.6. Statistical analysis

All experiments were replicated five times. Analysis of variance (ANOVA) of the data was performed using the software GraphPad Prism, version 5.01 (GraphPad Software, Inc., San Diego, CA, USA). Differences of percentages of morphologically normal preantral follicles (MNPF) and viable follicles between control and treatments (combinations of medium, temperature and time of storage) were determined by the Newman–Keuls' test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Experiment I: morphological evaluation of stored canine preantral follicles

3.1.1. Histological analysis

A total of 4045 preantral follicles were examined in histology, with a range of 146–179 in each treatment. Proportions of preantral follicles at each developmental stage were similar among fragments obtained from a pair of ovaries and thus follicular diameters were homogenous between treatments. Most of the studied follicles were at the primordial or resting stage and had a diameter of 25–30 µm. Primary or activated follicles (30–40 µm) and secondary follicles (40–100) were also analyzed in lower numbers equally found in all experimental groups. MNPF in control as well as after storage exhibited a spherical or elliptical oocyte with a large central or eccentric nucleus and uniform cytoplasm. Granulosa cells without pycnotic nuclei were well-organized in layers surrounding the oocyte and a distinguishable intact basement membrane could be observed (Fig. 2A–C). Degenerated follicles showed a retraced oocyte with or without a pycnotic nucleus, and occasionally a strongly eosinophilic cytoplasm (Fig. 2D). Layers of granulosa cells remained unaltered or became disorganized into a low density mass of cells which were many times swollen and/or detached from basement membrane. Occurrence of pycnotic bodies in granulosa cells or rupture of the basement membrane were not observed.

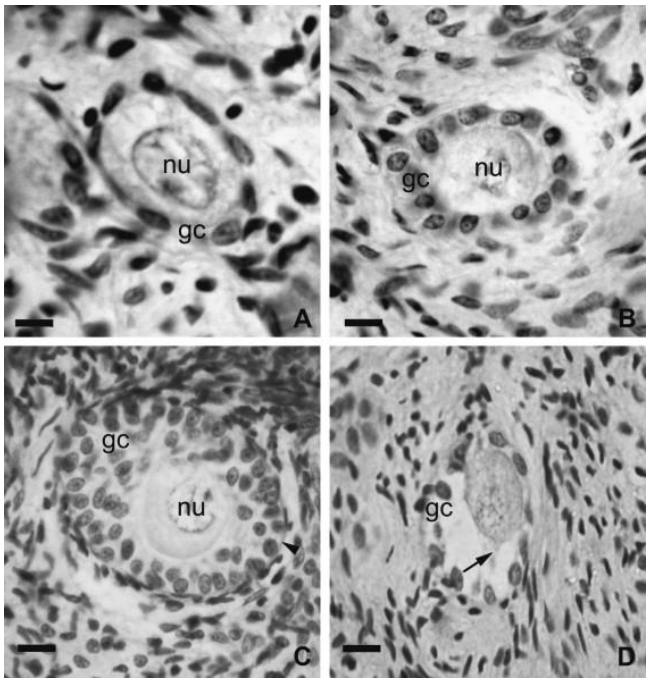

Fig. 2. Histological features of stored canine ovarian fragments. Morphologically normal primordial (A) and primary (B) follicles comprised an oocyte (o) displaying a large nucleus (nu) and homogenous cytoplasm surrounded by one layer of flattened or cuboidal granulosa cells (gc), respectively, whilst in secondary follicles two or more layers were present without antrum (C). Degenerated preantral follicles often displayed oocyte retraction (D, arrow) and disorganization of granulosa cell layers. The arrowhead indicates an intact basement membrane. Scale bars represent 6 µm (A) and 10 µm (B–D), PAS-hematoxilin.

The effects of medium, temperature and time of storage on the percentages of MNPF are shown in Fig. 3. Maintenance in saline solution at 4 °C for up to 24 h, at 20 °C for up to 12 h or at 38 °C for 6 h kept proportions of MNPF similar ($P > 0.05$) to control values (time zero). A decrease ($P < 0.01$) of the percentages of MNPF in relation to the control was observed after holding in saline solution at 20 °C for 24 h and at 38 °C for 12 and 24 h. With respect to MEM, similar results were obtained, with exception of the maintenance of follicles at 20 °C for 24 h and at 38 °C for 12 h, for which percentages of MNPF were also similar ($P > 0.05$) to control values.

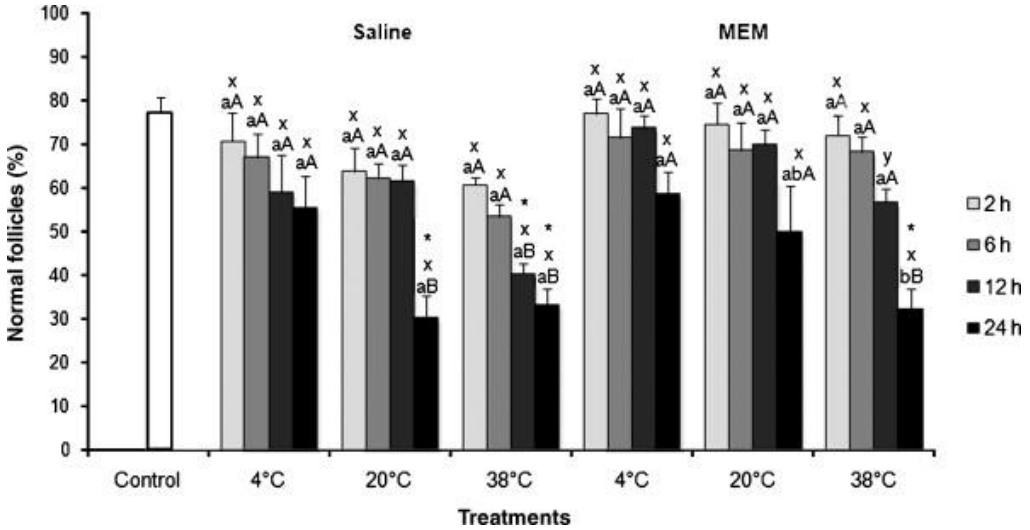

Fig. 3. Effects of medium, temperature and time of storage on the percentages of morphologically normal canine preantral follicles. *Differs significantly from the control ($P < 0.05$); (a–c) different letters for the same medium and time of incubation denotes significant differences between temperatures ($P < 0.05$); (A–C) different letters for a given combination of medium and temperature indicates significant differences among times of incubation ($P < 0.05$); (X, Y) different letters represent significant differences between media for fixed time and temperature of incubation.

The effect of storage time on the percentages of MNPF at each temperature and for each solution was analyzed. At 4 °C, percentages of MNPF were not affected by the incubation time, for both solutions. Similar results were obtained after preservation in MEM at 20 °C. Otherwise, when ovarian fragments were kept at 20 °C in saline solution, there was a decrease ($P < 0.01$) in the percentages of MNPF after 24 h compared to 2, 6 or 12 h. A reduction ($P < 0.05$) in the proportions of MNPF occurred at 38 °C with the increase of incubation time to 12 h in saline solution and to 24 h in MEM.

Regarding the effect of temperature on percentages of MNPF for a defined period of storage, a decrease ($P < 0.05$) in percentages of MNPF was observed when ovarian fragments were maintained in MEM for 24 h at 38 °C in comparison to 4 °C.

The comparison between saline solution and MEM at a same temperature and storage period showed that at 38 °C after 12 h, percentages of MNPF were higher when ovarian fragments were maintained in MEM.

3.1.2. Ultrastructure analysis

Transmission electron microscopy of stored preantral follicles was performed for assessment of ultrastructure in comparison to normal follicles from the control group. At least five follicles per group were analyzed. Normal follicles contained an oocyte displaying a very homogenous cytoplasm plenty of round shaped mitochondria with continuous membranes, few peripheral cristae and electron-dense granules. Some elongated forms with parallel cristae could also be seen. Small Golgi apparatus cisternae were rarely observed. Smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria. The nuclei of oocytes were large and usually round, well delimited by the nuclear envelope, the chromatin was uncondensed and a nucleolus could often be identified. Granulosa cells were small and presented a high nucleus-to-cytoplasm ratio. Their irregularly shaped nuclei contained loose chromatin in the inner part and small peripheral aggregates of condensed chromatin. The cytoplasm exhibited a great number of mitochondria and well-developed smooth and rough endoplasmic reticulum. The cellular membranes of oocyte and surrounding granulosa cells were closely justaposed, and sometimes few short microvilli could be observed. A distinct continuous basement membrane surrounded follicles and was tightly attached to ovarian stroma (Fig. 4).

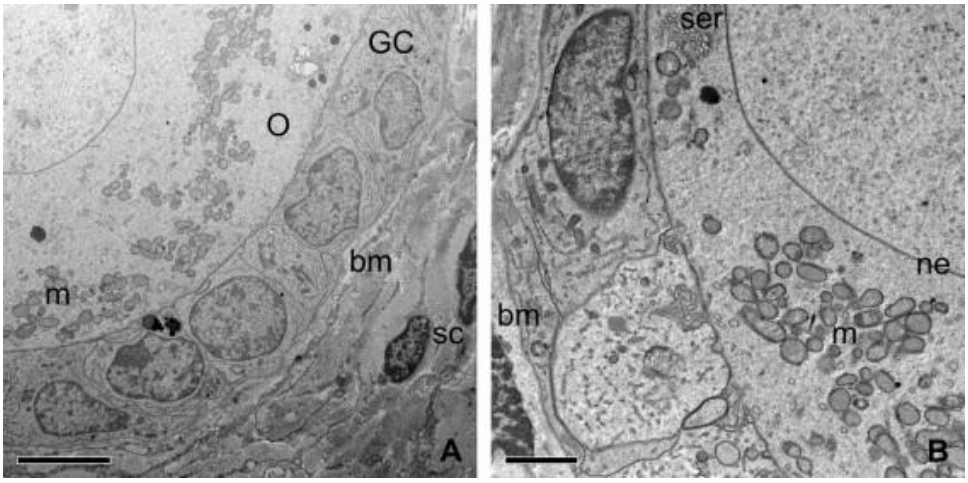

Fig. 4. Electron micrographs of normal follicles from control group (A) and stored in MEM at 4 °C for 12 h (B). O, oocyte; GC, granulosa cells; ne, nuclear envelope; m, mitochondria; ser, smooth endoplasmic reticulum; bm, basement membrane; sc, stromal cells. (A, 5000×, scale bar = 5 μm; B, 10,000×, scale bar = 2 μm).

The ultrastructural pattern described above was observed in normal follicles from control as well as after storage in MEM at 4 or 20 °C for up to 12 h and at 38 °C for up to 6 h; in saline solution at 4 or 20 °C for up to 6 h. When ovarian fragments were incubated at 4 °C in MEM or in saline solution for 24 and 12 h, respectively, and at 38 °C in saline solution for 2 h,

discreet changes in ultrastructure suggesting the onset of degeneration could be observed in follicles evaluated as morphologically normal in semi-thin sections by light microscopy. Some mitochondria both in the oocyte and in granulosa cells were very enlarged, displayed fewer cristae and lower electron density of the matrix, which may indicate swelling (Fig. 5).

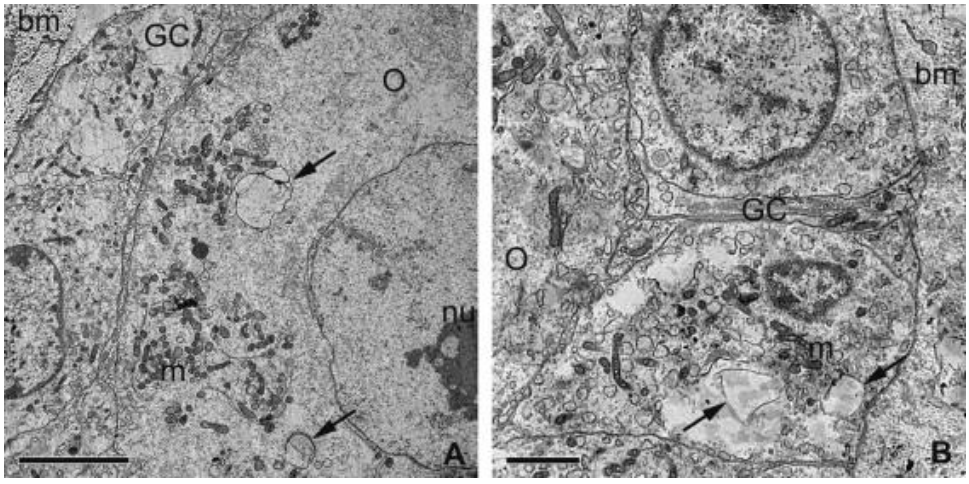

Fig. 5. Ultrastructure of follicles stored in MEM at 4 °C for 24 h. Mitochondria displaying substantial enlargement, loss of peripheral cristae and an electron-lucent matrix (arrows) were observed both in oocytes (A) and granulosa cells (B). O, oocyte; GC, granulosa cells; nu, nucleolus; m, mitochondria; bm, basement membrane. (A, 6000×, scale bar = 5 µm; B, 10,000×, scale bar = 2 µm).

More severe alterations of ultrastructure were noticed after storage in saline solution at 4 °C for 24 h, at 20 °C for 12 h and at 38 °C for 6 h; in MEM at 20 °C for 24 h and at 38 °C for 12 h. Follicles contained high numbers of vacuoles spread throughout the cytoplasm of all cells, which often fused creating large vacated areas. Furthermore, signs of proliferation of the endoplasmic reticulum and damage to mitochondrial membranes and cristae were observed. Granulosa cells had a swollen aspect and presented a very low density of organelles. Some of these cells completely disappeared, resulting in a empty space. In oocytes, swelling of the nuclear cisterna and disruption of the nuclear envelope were observed along some segments of the nuclear outline (Fig. 6). In some follicles kept in MEM at 20 °C for 24 h, the cytoplasm of oocyte and granulosa cells seemed to be clotted, possibly due to nuclear content leak.

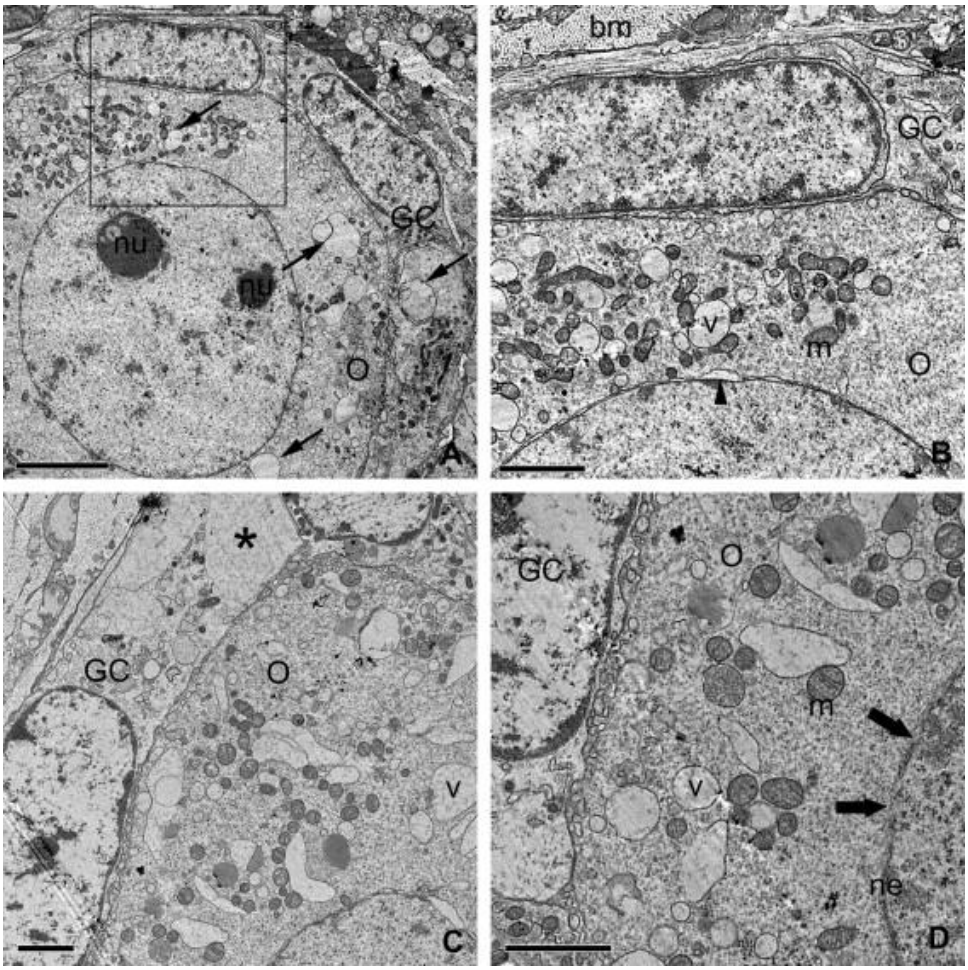

Fig. 6. Electron micrographs of follicles stored in saline solution at 4 °C for 24 h. A primordial follicle containing several vacuoles spread throughout the ooplasma as indicated by arrows (A, 5000×, scale bar = 5 µm); the inset is displayed at a higher magnification (B, 12,000×, scale bar = 2 µm), note the occurrence of nuclear cisterna swelling (arrowhead). Another primordial follicle exhibiting widespread vacuolization and a granulosa cell with a very large vacated area indicated by an asterisk (C, 8000×, scale bar = 2 µm); a higher magnification of the same follicle (D, 15,000×, scale bar = 2 µm), observe the discontinuity of the nuclear envelope (thick arrow). O, oocyte; GC, granulosa cells; nu, nucleolus; ne, nuclear envelope; m, mitochondria; bm, basement membrane.

3.2. Experiment II: assessment of viability of stored canine preantral follicles

Preantral follicles were mechanically isolated from control and stored canine ovarian fragments and viability was assessed by trypan blue dye exclusion test (Fig. 7). A total of 6037 follicles were examined, with a range of 171–281 in each treatment.

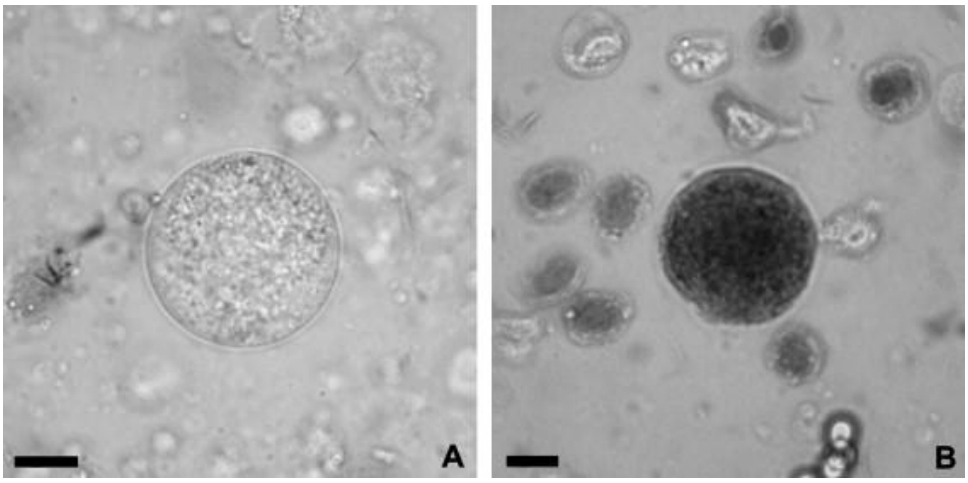

Fig. 7. Viability assessment of canine preantral follicles using trypan blue (TB) dye exclusion test. Isolated preantral follicles were incubated with 0.04% TB for 5 min and then classified as viable if the oocyte and <10% granulosa cells remained unstained (A), or as non-viable if oocyte and/or ≥10% granulosa cells were stained (B). Scale bars represent 10 μm.

The effects of medium, temperature and time of storage on the percentages of viable preantral follicles are shown in Table 1. Maintenance in saline solution at 4 or 20 °C for up to 6 h kept proportions of viable follicles similar ($P > 0.05$) to control values (time zero). A decrease ($P < 0.01$) of the percentages of viable follicles in relation to the control was observed after holding in saline solution at 4 or 20 °C for 12 and 24 h, and at 38 °C after all incubation times. With respect to MEM, storage of follicles at 4 or 20 °C for up to 12 h, and at 38 °C for up to 6 h preserved viable follicles, whose percentages were similar ($P > 0.05$) to control values.

Medium	Temperature (°C)	Time (h)			
		2	6	12	24
Control		67 (186/279)			
Saline	4	55% abA (151/276)	52% abA (132/255)	41%* abA (107/259)	37%* abA (87/234)
	20	54% abA (123/229)	52% abAB (145/281)	39%* abB (97/246)	20%* bC (35/171)
	38	43%* bA (96/222)	43%* bA (115/265)	30%* bB (69/231)	23%* bB (45/195)
MEM	4	67% aA (172/258)	61% aA (159/259)	64% cA (176/275)	48%* aB (96/200)
	20	65% aA (171/264)	58% abAB (160/277)	60% cAB (162/272)	32%* abB (59/185)
	38	62% aA (138/222)	58% abAB (148/255)	47%* aB (111/238)	22%* bC (42/189)

(a–c) Different letters denotes significant difference between values within a column ($P < 0.05$); (A–C) different letters indicates
significant difference between values of a row ($P < 0.05$).
* Differs significantly from control ($P < 0.05$).

The effect of storage time on the percentages of viable follicles at each temperature and for each solution was analyzed. At 4 °C, percentages of viable follicles in saline solution were not affected by the incubation time, but a significant reduction ($P < 0.05$) was observed in MEM from 12 to 24 h of holding. When ovarian fragments were kept at 20 °C in saline solution, there was a decrease ($P < 0.01$) of the percentages of viable follicles after 12 h, and a further reduction from 12 to 24 h; in MEM, this reduction was observed after 24 h only. A decrease (P

< 0.05) in the proportions of viable follicles occurred with the increase of incubation time to 12 h in both solutions at 38 °C. An additional decrease (P < 0.01) was observed in MEM at 38 °C from 12 to 24 h.

Regarding the effect of temperature on percentages of viable follicles for a defined period of storage, a reduction (P < 0.05) in the viability of follicles was observed when ovarian fragments were maintained in MEM at 38 °C for 12 h compared to 4 and 20 °C, and for 24 h, in relation to 4 °C only.

The comparison between saline solution and MEM at a same temperature and incubation period showed that after storage at 38 °C for 2 h, and at all temperatures for 12 h, percentages of viable follicles were higher when ovarian fragments were maintained in MEM.

Based on the results of morphological evaluation (experiment I) and viability assessment with trypan blue, which evidenced that storage in MEM at 4 °C is able to maintain viability of canine preantral follicles for the longer time (12 h) and to preserve ultrastructure more efficiently, as verified after 24 h of incubation, a second viability trial using this protocol was performed with five replicates. In addition to trypan blue testing, a fluorescence cell viability assay based on labeling of live and dead cells by calcein-AM and ethidium homodimer-1, respectively, was employed. Hoechst 33343 was integrated to the test to allow counting of total cell number for the calculation of the percentage of viable granulosa cells within each follicle (Fig. 8).

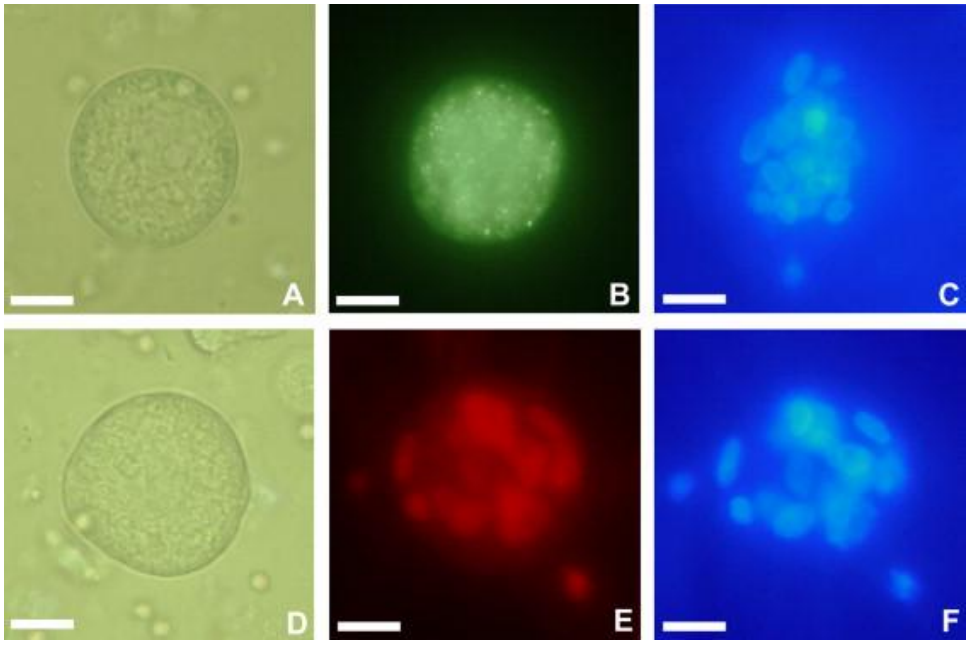

Fig. 8. Viability assessment of canine preantral follicles using fluorescent probes. (A) An isolated preantral follicle classified as viable (B) since cells were labeled by calcein-AM (green fluorescence). (D) A secondary follicle considered non-viable (E) as cells were marked with ethidium homodimer-1 (red fluorescence). (C and F) The same follicles in A and D, respectively, stained with Hoechst 33342, which was also used in the assay to allow counting of the total number of cells for calculation of the

percentage of dead cells. Scale bars represent 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

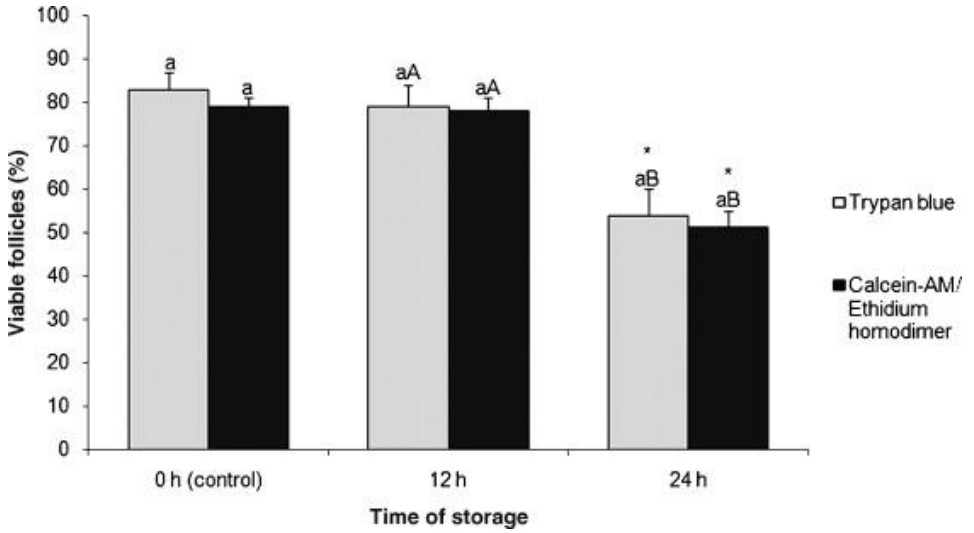

Fig. 9. Percentages of viable canine preantral follicles in fresh ovaries and after storage in MEM at 4 °C for 12 or 24 h as assessed by trypan blue dye exclusion test and labeling with calcein-AM and ethidium homodimer. *Differs significantly from control ($P < 0.05$); (a, b) different letters for values of viability assays within the same time of incubation denotes significant differences ($P < 0.05$); (A, B) different letters for results of a same test indicates significant differences between times of incubation ($P < 0.05$).

4. Discussion

The present study evaluated for the first time the short-term preservation of canine preantral follicles, which can be successfully accomplished using either 0.9% NaCl solution or MEM, but efficiency of each medium depends on temperature and time of holding.

Percentages of MNPF remained unaltered, as observed by histological analysis, after incubation at 4 °C in both media for up to 24 h, and at 20 °C in saline solution and MEM for up to 12 and 24 h, respectively. Conversely, holding at physiologic temperature (38 °C) maintained percentages of MNPF for only 6 h in saline solution and 12 h in MEM. Therefore, hypothermia during storage of canine ovaries provides more efficient preservation of morphology of preantral follicles. This may be explained by a reduction of metabolism, followed by a decrease in the requirements of oxygen and nutrients as well as metabolites production, which is critically important since cells are under ischemia. Indeed, hypothermia has proven to be an exceptional means of protection of any organ, with metabolic rate falling exponentially by about 50% per 6 °C drop in organ temperature (Kirklin and Barratt-Boyes, 1993). Our results are in accordance with other works, which reported that maintenance at 4 and 20 °C preserves morphology of bovine (Lucci et al., 2004), caprine (Carvalho et al., 2001, Ferreira et al., 2001, Silva et al., 2000 and Silva et al., 2001) and ovine (Andrade et al., 2001 and Matos et al., 2004) preantral follicles for longer times than at physiological temperature. In addition, Wood et al.

(1997) showed that maintenance in saline at 4 °C inhibited degeneration of cat follicles for 48 h after excision.

Transmission electron microscopy revealed that, after storage in saline solution at 4 and 20 °C for 12 h, at 38 °C for 2 h, in MEM at 4 and 20 °C for 24 h and at 38 °C for 12 h, preantral follicles considered morphologically normal in histology had alterations in ultrastructure indicative of degeneration. Therefore, electron microscopy proved to be a valuable tool to detect early morphological modifications due to atresia, which can be observed only at the ultrastructural level before becoming pronounced more grossly to be identified through light microscopy. Ultrastructure of ovine (Matos et al., 2004) and caprine (Silva et al., 2000) preantral follicles be preserved for up to 24 h at 4 °C. The shorter periods of preservation of ultrastructure observed in the present study may be due to a higher sensibility of canine preantral follicles to ischemia and/or to cold storage in relation to follicles of small ruminants.

Follicles in ovarian fragments maintained at 4 °C in MEM and saline solution for 24 and 12 h, respectively, and at 38 °C in saline solution for 2 h displayed the least degree of damaging, containing some enlarged mitochondria with reduced cristae and electron-lucent matrix. Silva et al. (2001) observed that the damage to mitochondria is one of the first signs of degeneration in goat preantral follicles during storage in vitro. Ischemia impairs cellular energetic metabolism which decreases the activity of the Na+/K+-ATPase pump with a gain in sodium (Bonz et al., 1998). During mild ischemia, mitochondrial matrix swells moderately due to the uptake of sodium (Garlid, 1996).

Incubation in MEM at 20 °C for 24 h and at 38 °C for 12 h, and in saline solution at 4 °C for 24 h and at 20 °C for 12 h led to more severe changes characterized mainly by high numbers of vacuoles spread throughout the cytoplasm of both oocyte and granulosa cells. Silva et al. (2001) demonstrated that structural changes due to degeneration progress with the increase of preservation temperature and incubation time. Atresia in vivo is also characterized by cytoplasmic vacuoles in oocytes (van den Hurk et al., 1998) and granulosa cells (Hay et al., 1976), which may be originated from endoplasmic reticulum swelling. On other hand, these vacuoles may be derived from altered mitochondria, as observed in cryopreserved bovine oocytes by Fuku et al. (1995).

Notwithstanding previous studies on preservation of preantral follicles provided important knowledge on this issue, approaches were limited to analysis of morphology, which is not always correlated with the viability of follicles (Santos et al., 2007). Therefore, in the present work, viability assessment using the trypan blue dye exclusion test was employed. It was observed that storage in MEM at 4 and 20 °C maintained percentages of viable preantral

follicles for up to 12 h, whilst at 38 °C such preservation was possible for up to 6 h. In saline, percentages of viable follicles were kept at 4 and 20 °C for up to 6 h, and at 38 °C, a significant decrease of viability was observed after only 2 h of incubation. Percentages of viable follicles were much lower than those of morphologically normal follicles in every treatment. This proves that a more precise determination of the proportions of follicles with adequate quality for subsequent applications such as in vitro culture may rely on viability assessment.

We further analyzed viability of canine preantral follicles stored in MEM at 4 °C, the protocol which maintained proportions of viable follicles for the longer time (12 h) whilst preserving ultrastructure more efficiently after 24 h, using a more accurate method based on fluorescent probes. For this purpose, labeling of non-viable cells with the disruption of plasma membrane was performed again by using ethidium homodimer-1, which enabled a better individualized visualization of dead cells through fluorescent staining of nuclei. Concomitantly, identification of viable cells was performed through detection of esterase activity with calcein-AM. Hoechst 33342 was used to mark all nuclei in each follicle for total cell number counting and calculation of percentages of live cells. Results of this assay were similar to values observed with trypan blue testing, which thus proved to be a reliable, practical and quick method for viability assessment of preantral follicles. Accordingly, Poeschmann et al. (2008) observed a significant correlation between both methods whilst analyzing viability of feline preantral follicles.

In this study, MEM was more efficient than saline solution to preserve viability of follicles after 12 h of incubation at any tested temperature. In addition, at 38 °C, MEM was able to maintain viability similar to control for 6 h, whilst, in saline solution, percentages of viable follicles were decreased after only 2 h of incubation. Furthermore, after 24 h at 4 °C, ultrastructure of follicles was altered to a lesser extent in ovarian fragments maintained in MEM. Therefore, composition of medium is critical for short-term storage of canine preantral follicles. We infer that endogenous nutrient resources in these follicles are very limited, thus it is necessary to provide supplementary nutritional compounds in preservation solutions. Although hypothermia may have reduced metabolic rates, these were possibly high enough to have depleted own energetic sources of follicles kept in saline at 4 and 20 °C after 6 h, leading to degeneration, whereas MEM, which comprises sugars, aminoacids, vitamins and inorganic salts, supported survival for up to 12 h.

In conclusion, preservation of canine preantral follicles during storage is achieved more efficiently through hypothermia in a nutritive medium. Maintenance of viability and ultrastructural integrity can be accomplished at 4 and 20 °C in saline solution for 6 h or in MEM for 12 h, being the later solution also adequate at 38 °C for 6 h. We suggest the use of MEM at

4 °C for up to 12 h in order to provide optimal preservation of quality of canine preantral follicles for subsequent applications such as in vitro culture.

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