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Cryopreservation and characterization of canine preantral follicles

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ABSTRACT

The aim of this study was to define the population, morphological and ultrastructural characteristics of bitch preantral follicles (PAFs) and to compare the effects on the morphology of PAF of two cryopreservation techniques - slow freezing (SF) and vitrification (V) - of bitches' ovarian tissue. The average population (number per ovary) of PAFs was 48,541 ± 18,366, where 94.25% were primordial (45,145 ± 16,076). The average diameter of the primordial follicles was 27.5 ± 4.2 μm. The overall percentage of morphologically normal PAFs was 93.66 ± 6.81% for the control group, 86.16 ± 11.05% after SF and 68.14 ± 12.75% after V. The percentage of normal primordial follicles was 96.69 ± 4.72% in control, 89.51 ± 10.39% in SF and 75.32 ± 9.23% in V. There was no significant difference in the overall percentage of normal PAFs among SF and the control. However, slow frozen follicles presented ultrastructural damage, while vitrified primordial and primary follicles were well preserved. In conclusion, although slow freezing seemed to be a good preserving method, vitrification was more effective than slow freezing in preserving the ultrastructure of primordial and primary follicles of bitches.

1. Introduction

Cryobiology has revolutionized studies on reproduction because it allows the preservation of genetic material from rare breeds, endangered species and animals of commercial interest. Cryopreservation of ovarian tissue has been attempted in many species like sheep [1,2], humans [3,4], cows [5,6], goats [2,7], pigs [8] and bitches [9] with some very promising results.

Nowadays, there are two main techniques for female gamete cryopreservation: slow freezing and vitrification. Slow-freezing is usually achieved by pre-equilibration with 1.5–2 M permeating cryoprotectant agent, such as dimethyl sulfoxide (Me2SO), and then frozen with a controlled cooling rate [10]. The physical definition of vitrification is the solidification of a solution at low temperatures without ice crystal formation. The phenomenon is based on an extreme increase in viscosity and requires very quick cooling rates and the use of high concentrations of cryoprotectants, which reduces ice crystal formation and increases viscosity at low temperatures [11]. The solid surface vitrification method has been more and more used, especially for ovarian tissue [12–14]. It has the advantage of using a lesser quantity of vitrification solution and a higher heat exchange by using a cold metal surface; moreover, a clean metal surface facilitates sterile handling of biological material [15].

Although ovarian tissue cryopreservation is widely exploited in some species, reports of ovarian tissue cryopreservation in dogs (*Canis familiaris*) are scarce. Recently, live births from domestic dog embryos produced *in vitro* cryopreserved or not were reported [16]. Concerning ovarian tissue, studies have been published regarding the viability of ovarian tissue transplantation after vitrification, and those papers made just a few considerations about the follicular morphology and the percentage of morphologically normal follicles after cryopreservation [9,17]. One study [18] investigated the effect of slow freezing of bitch ovarian cortex and reported 82% of morphologically normal follicles after thawing. Another one evaluated two different cryoprotectants (Me2SO or 1,3-propanediol -PROH) on slow freezing of bitch ovarian cortex and demonstrated the higher efficacy on survival of canine oocytes using Me2SO [19]. Despite that, no studies have directly compared two methods of cryopreservation for bitches' ovarian tissue: slow freezing (SL) and vitrification (V). The development of cryopreservation techniques for canine preantral follicles may be very useful for preserving canids. The domestic dog may be used as a model for future development of cryopreservation techniques for wild canids, and in fact, few studies in this area have already been done [20]. Moreover, dog breeding has received increasing investment by breeders in various countries. Conservation of genetic material will thus soon be an attractive option for highly valuable animals. In particular, guide dogs for people with visual impairment may be benefited [21].

In addition, the ovarian morphology of domestic bitches have not

Abbreviations: Preantral Follicles, PAF; Slow Freezing, SF; Vitrification, V; 1,3-propanediol, PROH; dimethyl sulfoxide, Me2SO

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been fully described in the literature and there is only one study reporting population of ovarian bitch's focused in compare the number of preantral follicles between the bitches at different reproductive stages [22]. Moreover, the population of preantral follicles has been estimated for different species, such as bovine (*Bos indicus*: 35,288 - [23]; *Bos Taurus*: 89,577 - [24], bubaline [25], ovine [26], caprine [27], domestic feline [28], capuchin monkey (*Cebus apella*) 108,216 [29], and humans [30]. Similarly, the morphometry and ultrastructure of preantral follicles, which are very important parameters for the knowledge of their physiology, have been described for caprine [27], bovine [31,32], bubaline [25], swine [33] and domestic feline [28]. However, these data have not been described for dogs yet.

The aim of this study was to estimate the population, describe the morphometric and ultrastructural characteristics of canine preantral follicles, and to compare the effects on the morphology of preantral follicles of cryopreserving bitch ovarian tissue by SF and V.

2. Materials and methods

2.1. Animals and ovary collection

Ovaries were collected from healthy non-pregnant female dogs, between 6 months and 4 years of age undergoing elective ovariohysterectomy procedure, at the Veterinary Hospital of the University or private clinics. All ovaries were washed in saline solution and transported to the laboratory at 36 °C.

The work was divided in two parts. First, 5 ovaries from 5 different bitches were used to estimate the population and to describe the morphometry and ultrastructure of preantral follicles. Then, another 10 ovaries from 5 bitches were used for the cryopreservation experiment.

2.2. Part 1 – preantral follicles population, morphometry and ultrastructure

2.2.1. Light microscopy

To estimate the population of preantral follicles 5 whole ovaries were fixed in Carnoy fixative for 4–6 h, dehydrated in ethanol, clarified with xylene and embedded in paraffin wax (Histosec, Merck, Darmstadt, Germany). Serial sections (5 μm thick) were cut and every 120th section was mounted and stained with hematoxylin and eosin and examined under a Leica DM500 (Wetzlar, Germany) light microscope. All follicles with a visible oocyte nucleus were counted and classified as primordial, primary or secondary according to their morphological characteristics.

In order to estimate the follicular population correctly, we used a correction factor described by Gougeon and Chainy [34], namely:

$$N1 = No.St.Ts/So.dn$$

Where: N1 = the total number of follicles calculated per class; No = the number of follicles observed in the ovary; St = the total number of sections in the ovary; Ts = the thickness of the section (μm); So = the total number of sections observed; and dn = the mean diameter of oocyte nucleus for each follicle class.

Images were captured with a digital CCD camera (Sony DXC-107A, Tokyo, Japan), and measurements were performed with the aid of morphometric analysis software (Image Pro-Plus 5.1, MediaCybernetics, Bethesda, MD, USA). Granulosa cell counts and diameter measurements of follicles, oocytes, and oocyte nuclei were always obtained from the equatorial section of primordial (n = 100), primary (n = 50), and secondary (n = 40) follicles.

2.2.2. Transmission electron microscopy

Small pieces of ovarian cortex (approximately 1.0 mm^3) were fixed in 2% paraformaldehyde (v/v) and 2.5% glutaraldehyde (v/v) in 0.1 M sodium cacodylate buffer (pH 7.3) and postfixed in 1% osmium tetroxide (v/v), 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. The samples were *in bloc* contrasted with 0.5% uranil acetate (w/v), and then dehydrated in acetone and embedded in Spurr resin. Semi-thin sections (3.0 μm) were stained with toluidine blue and examined under a light microscope to localize the follicles. Ultrathin sections (70 nm) were examined in a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan).

In the present study, 21 primordial, 10 primary and 5 secondary follicles were used to describe their ultrastructure. Characteristics of oocyte and granulosa cells cytoplasm, presence and distribution of organelles, nuclear, plasmatic and basal membranes and zona pellucida were observed.

2.3. Part 2 - ovarian tissue cryopreservation

Three fragments of cortex from each ovary, measuring 1.0 mm x 1.0 mm x 5.0 mm were cut and randomly allocated to control (1 fragment immediately fixed), slow freezing (1 fragment) or vitrification procedures (1 fragment).

Cryoprotectant solution for slow freezing was prepared in Minimal Essential Medium (MEM - Invitrogen, Grand Island, NY, USA), supplemented with 20% fetal bovine serum (MEM+), 1.5 M Me2SO (Sigma-Aldrich, St Louis, USA) and 0.4% sucrose (w/v). For vitrification two solutions were used: VS1, composed of 1 M Me2SO in MEM+, and VS2, containing 2 M Me2SO, 1 M acetamide (Sigma-Aldrich, St Louis, USA), and 3 M PROH (Vetec, Duque de Caxias, RJ, Brazil) in MEM+.

2.3.1. Slow freezing procedure

Slow freezing procedure was based on the method described by Borges et al. [8], with modifications. Cortex fragments were equilibrated in cryovials containing 1 ml cryoprotectant solution at 10 °C for 20 min. After that, the cryovials were placed in a programmable freezer (BIOCOM, Uberaba, MG, Brazil) previously cooled to 10 °C and then cooled at a rate of 1 °C/min to −7 °C. At this temperature manual seeding was performed. Then the temperature was reduced 0.3 °C/min down to −30 °C, and the cryovials were removed from the freezer, plunged into liquid nitrogen (−196 °C) and stored for at least one week. For thawing, the cryovials were exposed to room temperature for 10 s and then immersed in a water bath at 37 °C for a period sufficient for full melting. For cryoprotectant removal, each ovarian fragment was subjected separately to three washes in MEM containing decreasing concentrations of sucrose (0.4%, 0.2% and 0) and Me2SO (0.75 M, 0.325 M and 0), for 5 min each time.

2.3.2. Vitrification procedure

The method of vitrification was based on the method described by Ishijima et al. [9], with modifications. Initially, the fragments were kept in VS1 for 5 min and then on VS2, for another 5 min, always at 0 °C. The vitrification process was carried out on solid surface. The pieces of ovarian cortex were retrieved from VS2 and immediately dropped on a dry inox surface that was partially immersed in liquid nitrogen. After that, the samples were collected from the surface with precooled forceps and quickly placed in cryotubes previously cooled in liquid nitrogen, which were then immersed in liquid nitrogen and kept stored for at least a week. For warming, cryovials were exposed to room temperature for 10 s and then immersed in a water bath at 37 °C at the same time that 1.5 ml of warmed (37 °C) MEM+ with 0.25 M sucrose was added to the cryovial, and maintained for a period of 5 min. To remove the cryoprotectant solutions were changed twice, reducing the concentration of sucrose to 0.125 M and then 0 M, each time with a 5-min interval.

At the end of each treatment, each fragment was divided into two parts: a small sample (~1.0 mm^3) was immediately fixed and processed for transmission electron microscopy and the remaining part was fixed and processed for light microscopy.

For histological analysis, the ovarian pieces were fixed in Carnoy fixative for 1 h, dehydrated in ethanol, clarified with xylene and

embedded in paraffin wax. Sections (5 μm) were taken randomly and stained with hematoxylin and eosin and examined under a light microscope. At least 5 sections per fragment were analyzed. Follicles (with a visible oocyte nucleus) were counted and classified according to the stage of development (primordial, primary or secondary) and as morphologically normal or degenerated. Follicles were considered degenerated when presenting pyknotic bodies in granulosa cells, condensed oocyte nucleus, shrunken oocyte, oocyte cytoplasm vacuolization or low cellular density. The percentage of morphologically normal and degenerated follicles was calculated for each ovarian piece.

Morphometry was also performed for morphologically normal follicles after slow freezing and vitrification. Diameter measurements of follicles, oocytes, and oocyte nuclei were performed in the same way described for fresh ovaries, always obtained from the equatorial section of primordial (n = 100), primary (n = 50), and secondary (n = 40) follicles from each preservation method. At least 10 primordial, 5 primary and 4 secondary follicles were measured per ovary.

For ultrastructural analysis, the small pieces of ovarian cortex were processed as described above. Only follicles that were of normal morphology on semi-thin sections were evaluated. Characteristics of oocyte and granulosa cells, their organelles and membranes were observed. A total of 25 follicles were evaluated after cryopreservation (slow freezing or vitrification).

2.3.3. Statistical analyses

The diameter of follicles, oocytes and nuclei, and the number of granulosa cells, were compared among follicle classes by ANOVA and Tukey's test. The percentages of morphologically normal follicles were compared between the control and cryopreservation treatments. Data were transformed to Arcsin √% and submitted to ANOVA and Scheffé test. All analyses were performed using the software StatView for Windows (SAS Institute Inc., Cary, N.C., USA). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Follicular population and morphometry

The number of preantral follicles per ovary and the average diameter of the follicle, oocyte, oocyte nucleus, and the number of granulosa cells of bitch ovarian follicles are shown in Table 1. Significant differences ($P < 0.05$) were observed among the three classes for follicle, oocyte and oocyte nucleus diameter. However, only secondary follicles showed a significantly higher number of granulosa cells ($P < 0.05$) than primordial and primary follicles (Table 1).

3.2. Follicular ultrastructure

In the ultrastructural analysis, normal primordial follicles had an oocyte with homogeneous cytoplasm and a large round nucleus. The chromatin was loose and nucleoli were usually observed. The organelles were evenly distributed throughout the cytoplasm (Fig. 1A and C). The most abundant organelles were round mitochondria, but a few elongated mitochondria could also be seen. Rough endoplasmic reticulum cisternae were observed, in most cases associated with mitochondria (Fig. 1B). Golgi cisternae were sometimes found. The membranes of oocyte and granulosa cells were attached, with a close contact supported through invaginations (Fig. 1C). The granulosa cells were flat and small, with a large nucleus and cytoplasm with a few organelles (Fig. 1C).

Primary follicles' ultrastructure was very similar to that observed in the primordial stage (Fig. 1D). Oocytes were usually spherical, but elongated oocytes were occasionally observed. Round mitochondria were still the most abundant organelle. The endoplasmic reticulum and Golgi cisternae were also present (Fig. 1D). At this stage granulosa cells were cuboidal, their nuclei were round and they had a higher number of organelles, mainly mitochondria and endoplasmic reticulum (Fig. 1D).

Secondary follicles showed the beginning of zona pellucida formation around the oocyte, and oocyte microvilli started to penetrate it (Fig. 1E). More advanced secondary follicles had a completely formed zona pellucida and granulosa cells projections could be seen across it, along with oocyte microvilli (Fig. 1F). Round and elongated mitochondria could still be observed (Fig. 1E and F). Cisternae of endoplasmic reticulum were abundant and the association with mitochondria was common. Vesicles were abundant when compared to the previous stages. In secondary follicles, granulosa cells were cuboidal and had many organelles (Fig. 1E).

3.3. Cryopreserved follicles

A total of 1747 follicles were evaluated by light microscopy, (831 fresh, 591 frozen/thawed and 325 vitrified/warmed follicles). The percentage of morphologically normal follicles (Table 2), was significantly lower in the vitrification treatment when compared to control and slow freezing ($P < 0.05$), regardless of follicular class. There was no significant difference between control and slow freezing treatment.

The morphology of fresh control follicles (Fig. 2A–C) and frozen/thawed follicles (Fig. 2D–F) was very similar. The oocyte presented a well-defined homogeneous cytoplasm in close contact with granulosa cells. The most commonly observed characteristics of degeneration were pyknotic nuclei (Fig. 2G) and total disintegration of the oocyte (Fig. 2H), mostly after vitrification, but also after slow freezing. In vitrified ovarian tissue only, an interesting feature observed was the detachment of the whole follicle from the ovarian stroma (Fig. 2I), especially in secondary follicles. However, in most cases, the follicle itself showed normal morphology and was classified as such.

The average diameter of follicle, oocyte and oocyte nucleus of cryopreserved follicles is shown in Table 3. For secondary follicles, significant difference ($P < 0.05$) among treatments was observed both for follicle, oocyte and nucleus diameter. Slow freezing follicles always presented smaller sizes than the fresh follicles. Vitrified primary follicles showed oocyte and oocyte nuclei significantly smaller ($P < 0.05$) than fresh follicles. Primordial follicles had smaller oocyte nuclei after vitrification when compared to fresh and slow frozen follicles ($P < 0.05$).

The ultrastructural analysis showed divergent results compared to

Table 1
Mean number (± SD), percentage of each class and morphometric parameters (mean ± SD) of preantral follicles in bitches.

Class	Population (%)	Diameter (μm)			Number of granulosa cells
		Follicle	Oocyte	Oocyte nucleus	
Primordial	45,145 ± 16,076 (94.25)	27.5 ± 4.2[a]	21.7 ± 2.7[a]	11.3 ± 1.6[a]	6.0 ± 1.8[a]
Primary	2358 ± 1253 (4.92)	42.6 ± 12.5[b]	27.8 ± 7.5[b]	13.7 ± 2.6[b]	15.0 ± 7.0[a]
Secondary	397 ± 351 (0.83)	101.6 ± 62.9[c]	48.0 ± 12.6[c]	18.7 ± 4.2[c]	61.5 ± 63.5[b]
Total	47,900 ± 17,680	–	–	–	–

[a,b,c] Numbers with different letters in the same column differ statistically ($P < 0,05$).

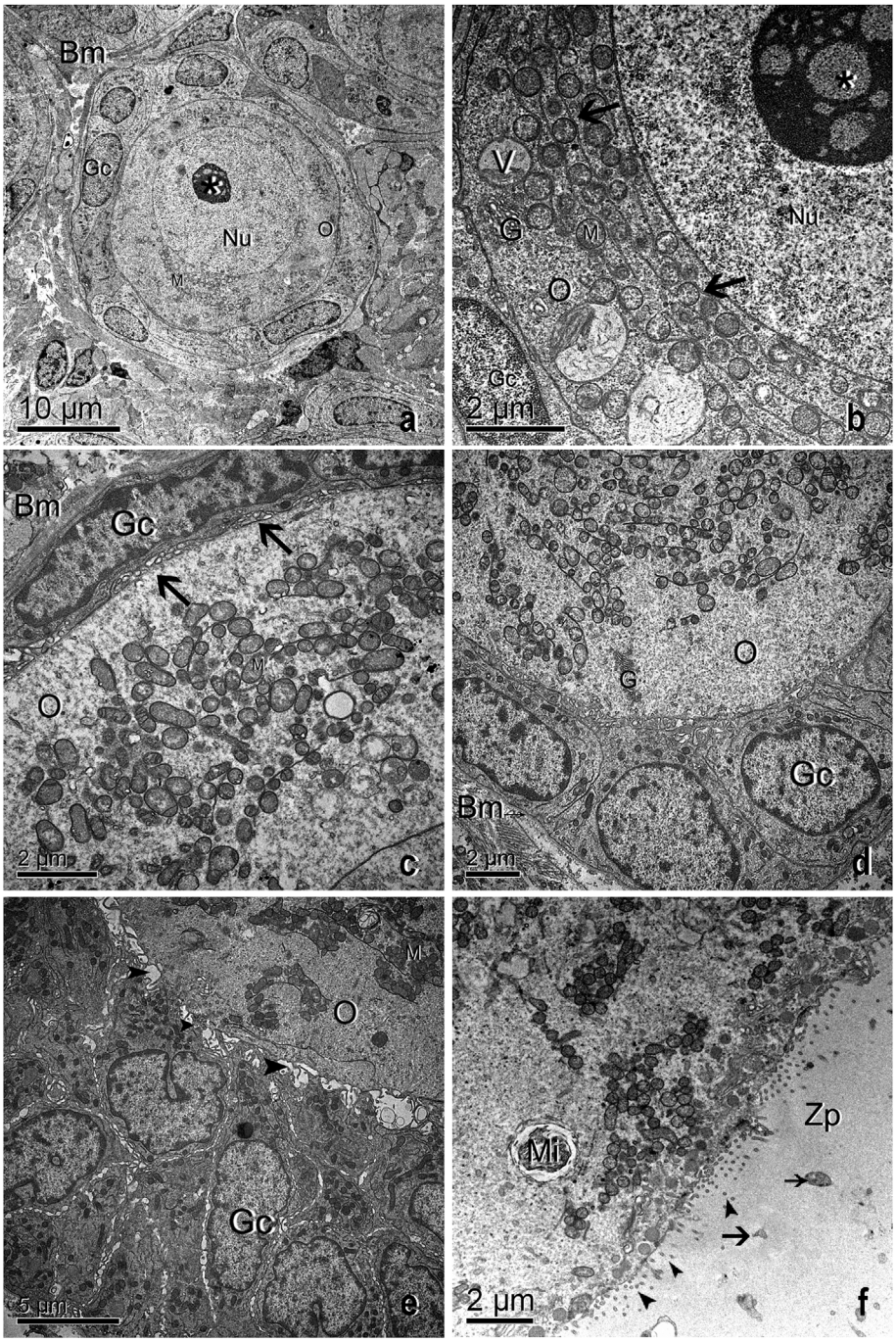

Fig. 1. Electron-micrographs of bitch preantral follicles. (*a*) Overview of primordial follicle. (*b*) Detail of the oocyte of a primordial follicle showing the association of mitochondria and endoplasmic reticulum (arrows). (*c*) Detail of contact between oocyte and granulosa cells in primordial follicles. Note the invaginations (arrows). (*d*) Primary follicle showing the association of mitochondria and endoplasmic reticulum, also seen in primordial follicles. Note the greater amount of granulosa cell organelles. (*e-f*) Secondary follicles showing either the zona pellucida in development (*e*) or completely formed around the oocyte (*f*). Note the oocyte microvilli (arrowheads) and granulosa cells projections (arrows). O: oocyte, Gc: granulosa cell, Nu: nucleus, Zp: zona pellucida, M: mitochondria, Mi: myelin figure, G: Golgi, *: nucleolus, Bm: basement membrane, V: vesicle.

Table 2
Percentage (mean ± SD) of morphologically normal preantral follicles in fresh (control), frozen or vitrified ovaries.

Class	Control (%)	Slow freezing (%)	Vitrification (%)
Primordial	96.69 ± 4.72[a]	89.51 ± 10.39[a]	75.32 ± 9.23[b]
Primary	94.80 ± 6.91[a]	86.8 ± 12.15[a]	61.53 ± 14.78[b]
Secondary	87.62 ± 17.12[a]	76.35 ± 26.34[a]	52.25 ± 22.13[b]
TOTAL	93.66 ± 6.81[a]	86.16 ± 11.05[a]	68.14 ± 12.75[b]

[a,b] Numbers with different letters in the same row differ statistically ($P < 0.05$).

light microscopy. Surprisingly, vitrified follicles showed subcellular structure closer to the control group than follicles subjected to slow freezing. In primordial (Fig. 3A) and primary (Fig. 3B) follicles cryopreserved by slow freezing the oocytes presented regions with low density of organelles, presence of vesicles, slight detachment of granulosa cells, and disintegration of some granulosa cells. The nuclei of the oocytes had an irregular shape (Fig. 3A, B and C) and sometimes the nuclear membrane was ruptured (Fig. 3C). Most mitochondria were swollen (Fig. 3C). Secondary follicles also showed a total loss of oocyte cytoplasmic structure (Fig. 3D and E). Some secondary follicles presented a shrunken aspect, comparable to dehydration of the tissue (Fig. 3F). It is important to remember that only follicles classified as morphologically normal in the semi-thin sections were evaluated by TEM.

On the other hand, vitrified primordial (Fig. 4A and B) and primary follicles (Fig. 4C and D) were ultrastructurally similar to the control (see Fig. 1). Briefly, oocyte and granulosa cells were in close contact with each other (Fig. 4A–C), and many intact round mitochondria, often in association with endoplasmic reticulum, could be seen in the

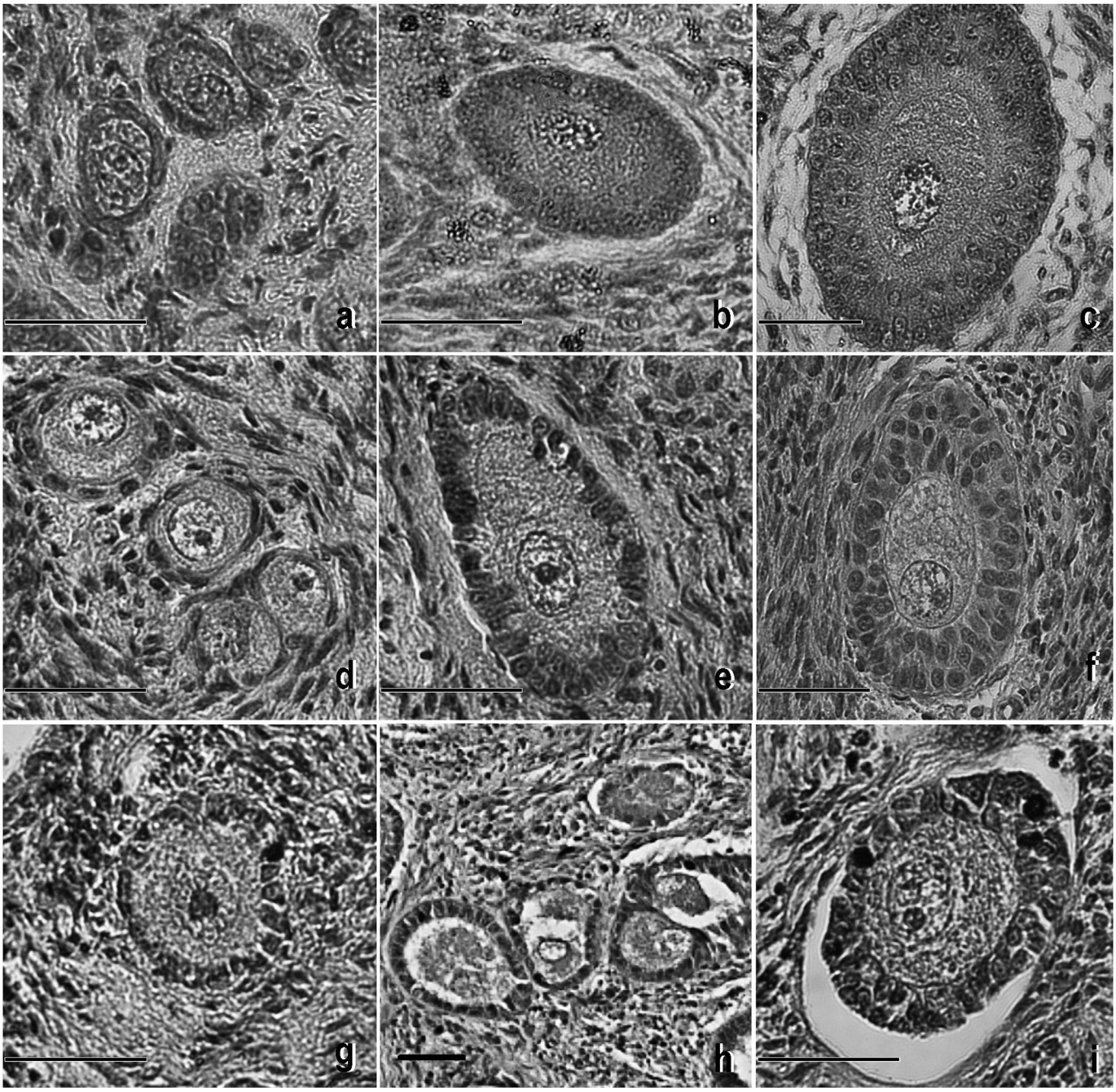

Fig. 2. Micrographs of morphologically normal primordial, primary and secondary follicles from the control (*a, b* and *c*, respectively) and slow freezing (*d-f*) groups. Degeneration signs mostly observed in vitrification group were pyknotic nuclei (*g*) and disintegration of oocyte (*h*). Morphologically normal secondary follicle detached from the ovarian stroma after vitrification (*i*). Bars = 20 μm.

Table 3
Mean morphometric parameters (mean ± SD) of cryopreserved preantral follicles in bitches.

Class	Diameter (μm)								
	Follicle			Oocyte			Oocyte Nucleus		
	C	SF	V	C	SF	V	C	SF	V
Primordial	27.5 ± 4.2	24.9 ± 4.8	27.5 ± 4.2	21.7 ± 2.7	19.9 ± 3.1	19.0 ± 2.9	11.3 ± 1.6^{a}	11.6 ± 2.3^{a}	9.5 ± 3^{b}
Primary	42.6 ± 12.5	34.5 ± 11.2	30.7 ± 3.7	27.8 ± 7.5^{a}	25.3 ± 6.1^{a,b}	22.6 ± 3.3^{b}	13.7 ± 2.6^{a}	12.5 ± 2.6^{a}	9.8 ± 1.8^{b}
Secondary	101.6 ± 62.9^{a}	55.8 ± 17.8^{c}	75.4 ± 23.3^{b}	48.0 ± 12.6^{a}	36.1 ± 9.3^{b}	42.9 ± 13.5^{a}	18.7 ± 4.2^{a}	14.7 ± 3.4^{b}	14.5 ± 4.3^{b}

C: Fresh control, V: Vitrification, SL: Slow Freezing. ^{a,b,c} Numbers with different letters in the same row differ statistically ($P < 0.05$).

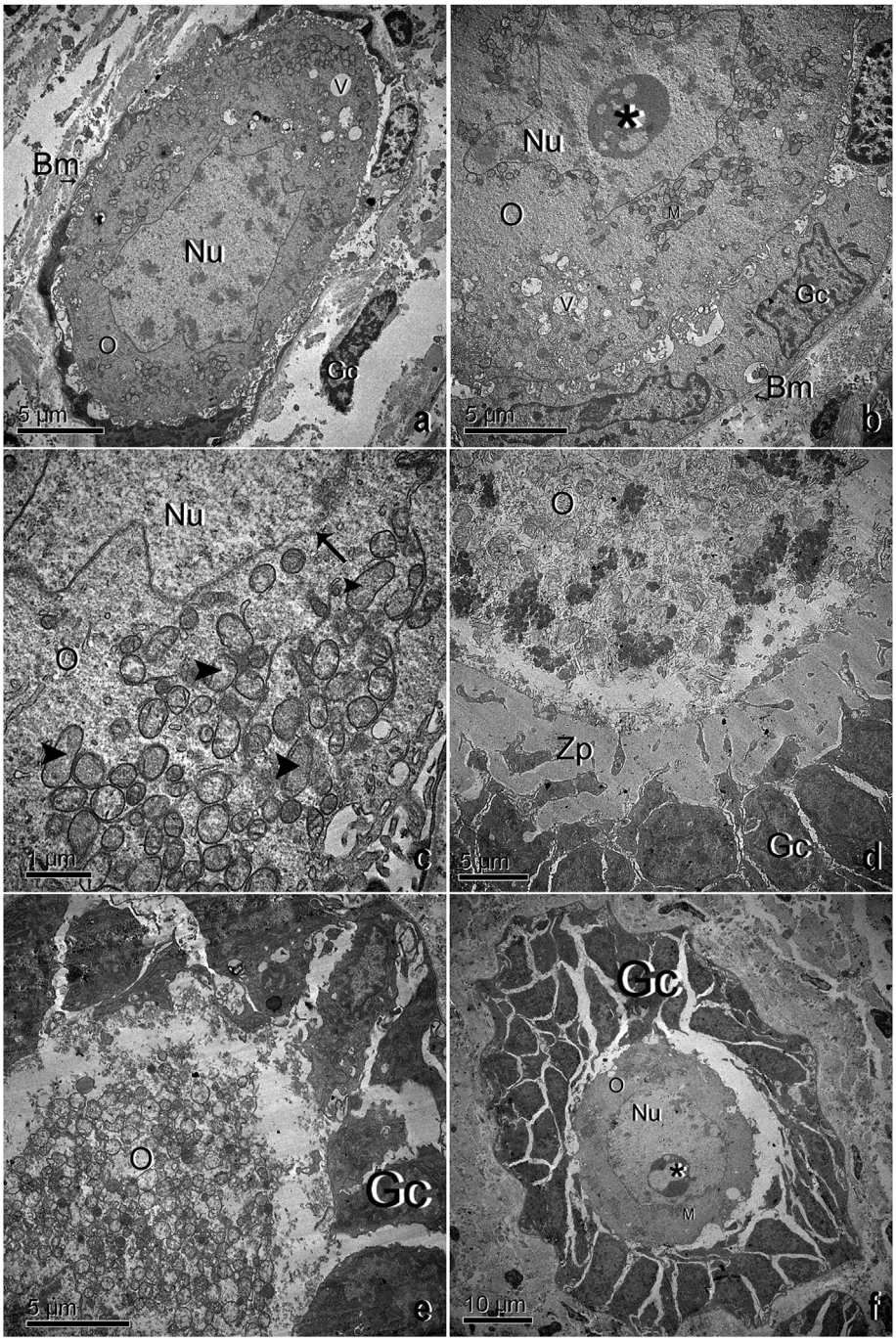

Fig. 3. Electron-micrographs of frozen/thawed preantral follicles. (*a*) Overview of primordial follicle. (*b*) Overview of primary follicle. (*c*) Detail of the oocyte of a primary follicle. Note the detachment of granulosa cells and the presence of vesicles and swollen mitochondria (arrowheads) in the oocyte. Oocyte nucleus with irregular contour and rupture of the nuclear membrane (arrow). (*d-f*) secondary follicles. Note the total loss of cytoplasmic structure (*d* and *e*) and the shrunken aspect (*f*). O: oocyte, Gc: granulosa cell, Nu: nucleus, Zp: zona pellucida, M: mitochondria, *: nucleolus, Mb: basement membrane, V: vesicle.

oocyte cytoplasm (Fig. 4D). In a single primordial follicle, a slight disruption of the nuclear membrane was observed (not shown). However, vitrified secondary follicles were not as well preserved. Their oocytes were vacuolated and presented a deformed nucleus (Fig. 4E) and swollen mitochondria without ridges (Fig. 4F). Even extensive degeneration of granulosa cells was observed (Fig. 4E).

4. Discussion

The estimated follicular population per ovary was approximately 47,900 preantral follicles. This number was similar to that described for the adult bitches (60,461) [22], domestic cat 37,853 [28] and goats 37,646 [27], but much smaller than that estimated in cows 750,000 [35], capuchin monkey (*Cebus apella*) 108,216 [29] and prepubertal bitches 107,152 [22] and higher than in buffaloes 19,819 [25]. Of all follicles, primordial follicles were the most abundant, corresponding to 94.25% of the follicular population. This high proportion of primordial follicles is consistent to that previously reported in bitches [22], queens [28], ewes [26] and cows [23]. Although in goats [27] the percentage of primordial follicles is not as high, they are still the vast majority (67.8%). A great difference, however, is shown in buffaloes [25] where approximately only 17.5% of the total population are primordial

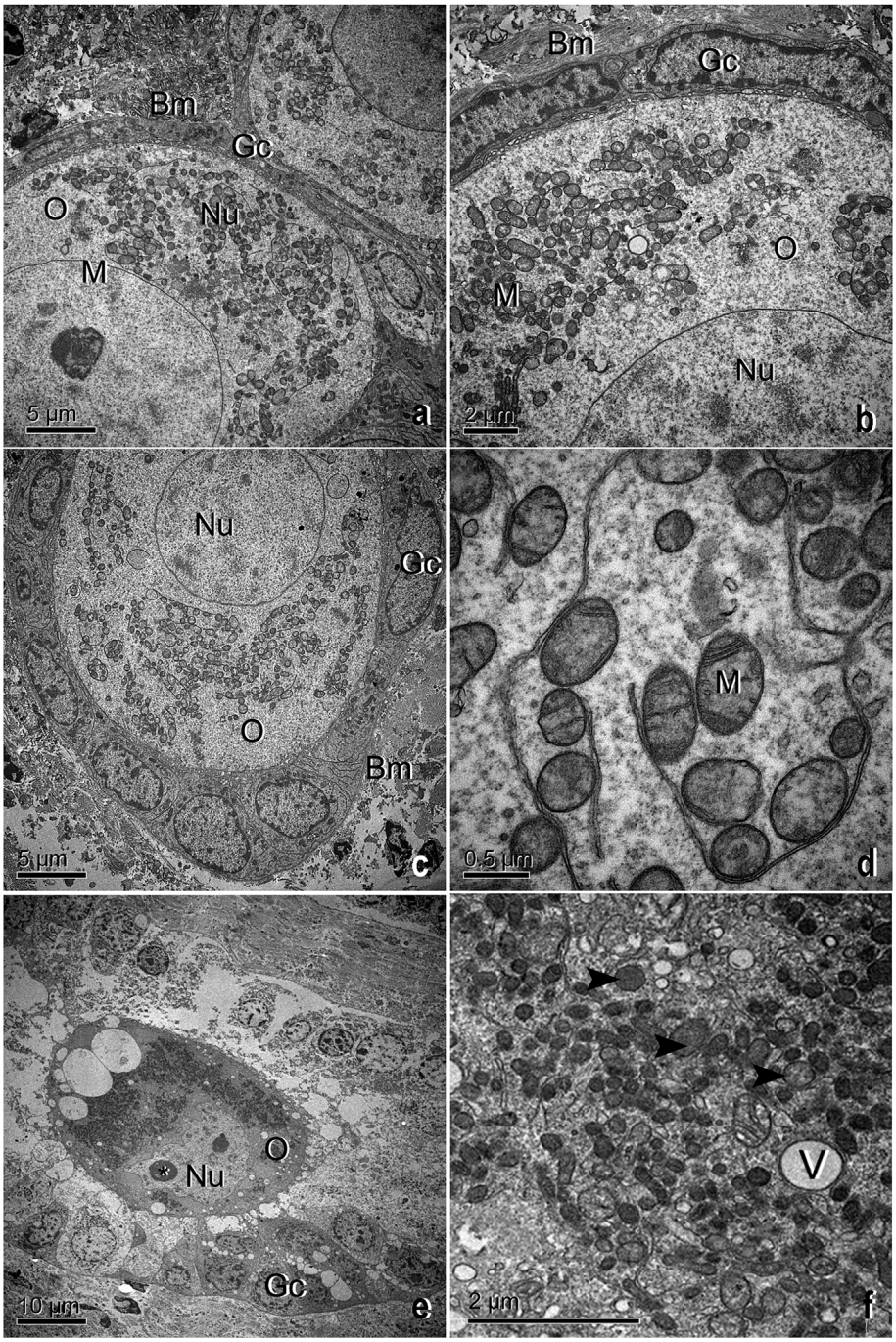

Fig. 4. Electron-micrographs of vitrified preantral follicles. (*a*) Overview of primordial follicles. (*b*) Detail of the oocyte of a primordial follicle. (*c*) Overview of primary follicle. (*d*) Detail of the oocyte of a primary follicle. Note the well-preserved ultrastructure of the oocyte with round well-delimited nucleus, round mitochondria in association with endoplasmic reticulum, homogeneously distributed throughout the cytoplasm. (*e*) Secondary follicle, showing extensive degeneration of granulosa cells and oocyte with vacuoles in the cytoplasm and deformed nucleus. (*f*) Detail of a secondary follicle oocyte cytoplasm showing swollen mitochondria without cristae (arrowhead). O: oocyte, Gc: granulosa cell, Nu: nucleus, M: mitochondria, *: nucleolus, Bm: basement membrane, V: vesicle.

follicles, and the vast majority (74%) are primary follicles.

Regarding follicle population, other studies reported bovine follicle population using 6 ovaries [23], bubaline follicle population using 5 ovaries [25], monkey follicle population using 3 ovaries [29] and cat follicle population using 5 [28]. Other studies were also carried out in other species using a higher number of ovaries, but the variation was still high. Lunardon et al. [22], reported the follicle population of 20 prepubertal medium-size dogs and found 135,467 follicles on average with 97,127 of standard deviation. We consider the individual variation natural and it will always happen, even if the number of ovaries evaluated is high.

The morphometry of bitch PAF was described in two studies [36,37]. The diameter of bitch primordial follicles found here (~25 µm in diameter), was similar to that reported by Durrant et al. [37]. The comparison for primary and secondary follicles was not possible since in that study the primary and secondary follicles were grouped into a single class (advanced preantral follicles). Furthermore, our results were quite different from those described by Diagone et al. [36], who reported measurements consistently higher than those obtained in this study. However, a hypothesis cannot be drawn about why this difference happened. Differences in follicular morphology within species are also seen among published studies for domestic cats [28,38]. In this case, the data from this study are comparable to those described by Carrijo Júnior et al. [28], but show lower values than those presented by Reynaud et al. [38]. Compared even with other species, the measurements obtained for dogs are similar to those reported for cattle [32], but higher than those described for buffaloes [39]. Knowledge of follicular morphometry is important for monitoring follicular growth in techniques such as *in vitro* culture of preantral follicles. Regarding the morphometry of slow frozen and vitrified follicles, the differences do

not seem to be connected to the treatment to which the ovarian tissue was subjected. It is quite expected that the diameter of cryopreserved follicles (and their oocytes) are smaller, since they were immersed in hyperosmotic solutions, especially immediately after thawing/warming. However, to our knowledge, there are no studies describing the morphometry of cryopreserved follicles, specially in bitches. Concerning stromal cells, a subjective analysis was performed and in the slow frozen tissue there were a higher density and well-preserved stromal cells than in vitrified tissue. Numerous recent studies have investigated stromal tissue density as an indicator of tissue integrity (reviewed by Shi et al. [40]).

The ultrastructure of primordial follicles observed in our study was similar to that previously described for dogs [41]. Lopes et al. [41] also described a general appearance of the follicles, but did not describe the characteristics of each class in detail. In our study, all classes of PAFs from bitches have been described. In general, the ultrastructure of bitches' PAFs is similar to that of other species (bovine [32], bubaline [39], ovine [42], feline [28] and swine [43].

Besides the follicular characterization, this study describes the effects of cryopreservation of bitches ovarian tissue on PAFs. Some other studies involving conservation (but not cryopreservation) of ovarian tissue have been carried out in dogs [41,44], demonstrating that dog PAFs can maintain their normal morphology when subjected to low temperatures (0-4 °C) for up to 36 h.

This study reports that the slow freezing of ovaries in bitches using Me2SO as cryoprotectant resulted in 86% of morphologically normal PAFs after thawing. These results are very similar to those of Commin et al. [18], which found 82% of morphologically normal follicles after a similar slow freezing procedure and to Lopes et al. [19], which found 84% of morphologically normal follicles using Me2SO and 80% using PROH as cryoprotectants after slow freezing. Me2SO has been proved to be a good cryoprotectant for slow freezing of pigs' [45] and cows' [5] ovarian tissue, with 72% and 74–88% of morphologically normal preantral follicles, respectively. On the other hand, studies in goats [7] and rabbits [46], using this cryoprotectant, showed a smaller percentage of morphologically normal preantral follicles, 48% and 50% respectively. In sheep, slow freezing of isolated primordial follicles using Me2SO resulted in 87% viable follicles [47]. All these results suggest a species-specific effect of the cryoprotectant.

This is also the first study that reports the effect of the bitch's ovarian tissue vitrification on the morphology of PAFs. Vitrification of canine PAFs has been already reported by Ishijima et al. [9] and Suzuki et al. [17], but the focus of those studies was the success of ovarian tissue transplantation after cryopreservation, and the percentage of PAF that maintained their normal morphology after cryopreservation was not mentioned. The vitrification of canine ovarian tissue by the solid surface method was used for the first time in this study, resulting in 68% of morphologically normal follicles in light microscopy analysis with well-preserved ultrastructure. The solid surface vitrification method was first described for bovine oocytes [15]. Using this method to preserve goat ovarian tissue, 70–80% of PAFs were histologically normal after warming [14]. Another study compared different methods of vitrification of goat ovarian tissue and described the solid surface as the most effective method, resulting in 72% of morphologically normal follicles [48]; a similar result was found in our study (68%). Vitrified canine cumulus-oocyte complexes (obtained from antral follicles) resulted in ~60% of oocytes with normal morphology (under stereomicroscope), but with low viability (5–17%, by propidium iodide) [49]. The well preserved ultrastructure of vitrified follicles (similar to control) could be an important indicator of the follicles' viability because it demonstrates the integrity of the organelles, suggesting a preservation of its function.

Evaluated by light microscopy, the rates of morphologically normal follicles were higher in slow freezing (86%) than in vitrification (68%), although both rates are quite satisfactory for cryopreservation procedures. Even though slow freezing did not cause many morphological changes in the histology, several signs of damage was found in the ultrastructure, such as mitochondrial swelling, low density of organelles in the oocyte cytoplasm, oocyte retraction and loss of granulosa cells. Similar ultrastructural injuries were reported for cryopreserved sheep PAFs [42]. The differences found between histological and ultrastructural evaluations using slow freezing have also been reported by other authors [8,42,50]. This probably happens because the slow freezing process is less aggressive, triggering minor osmotic stress on the cells and, consequently, the damage caused is inconspicuous, only visible under a higher resolution microscope which permits the long-standing effect of the cryoprotectant to be evaluated. However, using slow freezing in bitches Lopes et al. [19] found that TEM revealed ultrastructure alterations only in follicles frozen with PROH when compared to Me2SO. The higher degeneration rates observed by light microscopy after vitrification may be due to the very fast and aggressive procedure that uses very high concentrations of cryoprotectants. It is worth mentioning again that vitrified PAF did not present ultrastructural damages, and vitrified primordial and primary follicles showed similar characteristics to control follicles.

On the other hand, the ultrastructure of secondary follicles was damaged in both treatments. A study comparing slow freezing and vitrification techniques in rhesus monkey ovaries [51] also showed that secondary follicles were more likely to be damaged than primordial and primary follicles, in both treatments. The authors also reported that the vitrification treatment resulted in greater follicle integrity than slow freezing. In mice, secondary follicles cryopreserved by slow freezing or vitrification showed similar lesions in ultrastructure, such as mitochondrial deformities and a large number of vacuoles [52]. Moreover, electron microscopy is an essential tool to detect damage due to cryopreservation process, visible only at the ultrastructural level.

In conclusion, this work is the first to describe the follicle population and to compare the techniques of slow freezing and vitrification of bitch's ovarian tissue. The vitrification method was more effective in preserving the ultrastructure of primordial and primary follicles. For both treatments, improvements are needed in order to better preserve secondary follicles.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2018.02.013.

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